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(54) **Peptides Recognized by Melanoma-Specific Cytotoxic  
Lymphocytes, and Uses Therefor**

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US95/01991 <b>(22) International Filing Date:</b> 16 February 1995 (16.02.95)  <b>(30) Priority Data:</b> 08/197,399 16 February 1994 (16.02.94) US 08/234,784 29 April 1994 (29.04.94) US  <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 08/234,784 (CIP) Filed on 29 April 1994 (29.04.94)  <b>(71) Applicant (for all designated States except US):</b> UNIVERSITY OF VIRGINIA PATENT FOUNDATION [US/US]; Suite 1-110, Towers Office Building, 1224 West Main Street, Charlottesville, VA 22903 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SLINGLUFF, Craig, L. [US/US]; RR7, Box 168, Charlottesville, VA 22901 (US). ENGELHARD, Victor, H. [US/US]; 1401 Old Ballard Road, Charlottesville, VA 22901 (US). HUNT, Donald, F. [US/US]; 970 Old Ballard Road, Charlottesville, VA 22901 (US). SHABANOWITZ, Jeffrey [US/US]; Rt. 12, Box 64,		<div style="text-align: center; font-size: 1.2em;">2182889</div> <b>Charlottesville, VA 22901 (US). COX, Andrea, L. [US/US];</b> <b>502 Rugby Road, Charlottesville, VA 22901 (US).</b>  <b>(74) Agent:</b> COOPER, Iver, P.; Browdy and Neimark, 419 Seventh Street, N.W. #300, Washington, DC 20004 (US).  <b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>  <b>(88) Date of publication of the international search report:</b> 7 March 1996 (07.03.96) <div style="text-align: center; font-size: 1.2em;">2182889</div>
<b>(54) Title:</b> PEPTIDES RECOGNIZED BY MELANOMA-SPECIFIC CYTOTOXIC LYMPHOCYTES, AND USES THEREFOR		
<b>(57) Abstract</b>  Melanoma-specific CTL epitopes have been identified through improvements in the art of identification of epitope-reconstituting peptides dissociated from MHC Class I molecules on the surface of the tumor cells. Peptides homologous with a segment of the melanoma/melanocyte antigen pMel-17 have proven to be highly potent stimulators of HLA-A2*CTL in several CTL cell lines. These and kindred peptides may be used to stimulate CTLs for use in adoptive immunotherapy, or incorporated into immunogenic conjugates for use as vaccines.		

**PEPTIDES RECOGNIZED BY MELANOMA-SPECIFIC CYTOTOXIC  
LYMPHOCYTES, AND USES THEREFOR**

5

in their entirety.

**BACKGROUND OF THE INVENTION**

Field of the Invention

10       The present invention is directed to peptides that, in association with Class I MHC molecules, form epitopes recognized by cytotoxic T-cells specific for human melanoma, to immunogens comprising said epitopic peptides, and to related compositions, methods and apparatus.

15       Description of the Background Art

      Melanoma affects 30,000 new patients per year in the United States. It is a cancer manifested by the unabated proliferation of melanocytes. Eighty percent of melanoma patients are diagnosed during their productive years between  
20   the ages of 25 and 65. The incidence of melanoma is rapidly increasing, in 1935 the lifetime risk of developing melanoma was 1:1,500 individuals, at present, the risk has risen to 1:105. It is believed that by the year 2000 the risk of developing melanoma will increase to about 1:70 to 1:90.  
25   Early diagnosis and treatment of this disease is crucial. Once a primary tumor becomes metastatic the disease is almost always fatal.

      Cytotoxic lymphocyte (CTL) response has been shown to be an important host defense against malignant cells, Rock et  
30   al. J. Immunol., (1993), 150:1244.

      Lymphocytes isolated from patients having melanoma, when stimulated in vitro with recombinant interleukin-2 (rIL-2) and autologous melanoma cells, develop a melanoma specific cytotoxic response, Vose et al., Nature, (1982), 296:359;  
35   Knuth et al., Proc. Natl. Acad. USA, (1984), 81:3511; Slingluff et al., Arch. Surg., (1987), 122:1407; Darrow et al., Cancer, (1988), 62:84; Slingluff et al., J. Natl. Cancer Inst., (1988), 80:1016; Slingluff et al., Ann. Surg., (1989),

210:194; Muul et al., J. Immunol., (1987), 138:989; Van den Eynde et al., Int. J. Cancer, (1989), 44:634; Anichini et al., Int. J. Cancer, (1985), 35:683. The majority of melanoma-specific effector lymphocytes are CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) that are restricted by class I Major Histocompatibility Complex (MHC) molecules, Vose et al; Slingluff et al (1988), supra, Hersey et al., Cancer Immunol. Immunother., (1986), 22:15. These characteristics are present whether CTL have been generated from peripheral blood lymphocytes (PBL), lymph node cells, or tumor infiltrating lymphocytes.

The evidence that the CTL response to human melanoma is restricted by class I MHC molecules includes demonstration of cross-reactivity for allogenic melanoma cells that share a restricting class I MHC molecule with the autologous tumor. The HLA-A2 molecule and its variants, of which HLA-A2.1 is by far the most common, is an effective restricting element for the melanoma-specific CTL response. Additionally, melanoma-specific HLA-restricted CTL lyse the majority of A2<sup>+</sup> melanomas tested, Darrow et al., J. Immunol., (1989), 142:3329; Wolfel et al., J. Exp. Med., (1989), 170:797; Hom et al., J. Immunother., (1991), 3:153. By demonstrating lysis of A2- melanomas transfected with the A2.1 gene, it has been shown that these transfected melanomas can present the epitopes recognized by A2-restricted melanoma-specific CTL, Kawakami et al., J. Immunol., (1992), 148:638. These results suggest that these CTL recognize A2-restricted epitopes that are shared by the majority of melanomas, although very little is known about the number and identity of their epitopes.

Class I molecules of the Major Histocompatibility Complex (MHC) bind to peptides derived from intracellular pathogens or from proteins expressed in tumor cells, and present them on the cell surface to the host immune system. The mechanism of peptide presentation involves protein synthesis and proteolysis in the cytosol, followed by transport of peptides into the endoplasmic reticulum (ER), through the action of the TAP transporter molecules. Peptides then become associated with newly synthesized class

1 molecules, and the resulting complexes move to the cell surface. Proteins that are membrane associated or secreted contain signal sequences that cause them to be contraslationally transferred into the ER from membrane-bound  
5 ribosomes. Such proteins would thus be protected from the action of cytoplasmic proteases. However, since peptide epitopes do arise from such proteins, although their TAP dependent expression is unclear, it has been assumed that the proteolysis to generate these peptide epitopes occurs after  
10 these proteins have been aberrantly translated on cytoplasmic ribosomes.

Adoptive transfer of tumor stimulated CTL has been associated with some tumor regressions, Rosenberg et al., N. Eng. J. Med., (1988), 319:1676.

15 An alternate approach to augmenting the T-cell response to melanoma is the use of a vaccine to stimulate CTL in vivo (active specific immunotherapy). Epitopes for CD8<sup>+</sup> CTL are believed to be short, usually 9- residue peptides that bind to a cleft on the surface of the class I MHC molecule, Udaka  
20 et al., Cell, (1992), 69:989; VanBleek et al., Nature, (1990), 348:213; Falk et al., J. Exp. Med., (1991), 174:425. These peptides, generated from proteolysis of endogenous proteins in the cytosol, are transported to the endoplasmic reticulum, where they become associated with newly syn-  
25 thesized class I MHC molecules. They are then transported to the cell surface, Elliott et al., Nature, (1990), 3348:195. CTL epitopes have been reconstituted in vitro by allowing exogenous peptides to bind to MHC molecules on the cell surface of target cells, Townsend et al., Annu. Rev.  
30 Immunol., (1989), 7:601. However, because of the complexity of the peptide mixture associated with class I MHC molecules, Hunt et al., Science, (1992), 255:1261, the definition of individual peptides that comprise specific CTL epitopes has proven extremely difficult.

35 One method has been to generate genomic or cDNA libraries from tumor cells followed by transfection of progressively smaller subsets of these molecular clones into cells that express the appropriate MHC molecule, but not the

tumor specific epitope. Molecular clones that encode T cell epitopes are identified by their ability to reconstitute tumor-specific T cell recognition of the transfected cells. The exact T cell epitope is then identified by a combination of molecular subcloning and the use of synthetic peptides based on the predicted amino acid sequence. See, e.g., P. van der Brugge, et al., Science 254, 1643 (1991); C. Traversari, et al., J. Exp. Med. 176, 1453 (1992); B. Gaugler, et al., ibid. 179, 921 (1994); T. Boon, et al., Annu. Rev. Immunol. 12, 337 (1994); A.B.H. Baker, et al., J. Exp. Med. 179, 1005 (1994); Y. Kawakami, et al., Proc. Natl. Acad. Sci. USA 91, 6458 (1994); P.G. Coulie, et al., J. Exp. Med. 180, 35 (1994); Y. Kawakami, et al., ibid. 180, 347 (1994); V. Brichard, et al., ibid. 178, 489 (1993); T. Wolfe, et al., Eur. J. Immunol. 150, 2955 (1993). Unfortunately, it is possible to inadvertently identify clones that encode cross-reacting peptides that are recognized because of their high level of expression in the transfectants.

By this genetic method, an HLA-A1 restricted T cell epitope (EADPTGHSY) of a melanoma-associated antigen, MAGE-1, was identified. Traversari, et al., J. Exp. Med., 176:1453-57 (1992). MAGE-1 is expressed in about 20-40% of cancers of several different tissue types, including melanomas, breast cancers, non-small cell lung cancers, head and neck squamous cell cancers, and bladder cancer. It is also found in the normal male testis. The MAGE gene family also includes another member, MAGE-3, for which a homologous HLA-A1-restricted CTL epitope (EVDPIGHLV) was determined, although only after the first priority date. HLA-A1-restricted CTL epitopes are of limited utility because only a minority of melanomas are HLA-A1<sup>+</sup>. The function of the MAGE gene products is not known.

The genetic approach has also been used to identify HLA-A2.1-restricted CTL epitopes on tyrosinase. This enzyme is not tumor-specific; it is expressed by normal melanocytes as well as melanoma cells. Tyrosinase is involved in melanin biosynthesis. Autologous CTL recognized tyrosinase-derived

HLA-A2-restricted epitopes (YMNGTMSQV and MLLAVLYCL). See Wolfel, et al., Eur. J. Immunol., 24:759-64 (1994). However, these peptides were not recognized by the other CTL lines tested.

5 Another tissue-specific protein, gp100, is the target of the antibody HMB45, which is specific for melanoma and melanocytes. Based on the correlation between HMB45 activity and recognition by a single TIL-derived HLA-A2-restricted melanoma-specific CTL line, Bakker, et al., J. Exp. Med.,  
10 179:1005-9 (1994) established that transfection of cells with the gene for gp100 reconstituted the epitope recognized by this T cell. A subsequent study, using the same T-cell line to screen transfected cDNA libraries also identified the peptide LLDGTATLRL as being sufficient to reconstitute  
15 activity. This study was not published prior to Applicants' first priority date. Gp100 is believed to play a role in melanin biosynthesis.

An HLA-A2.1-restricted epitope (AAGIGILTV) has also been identified genetically in another melanocytic protein, MART-1  
20 (Melan-A). Kawakami, et al., J. Exp. Med., 180:347-52 (1994) and Proc. Nat. Acad. Sci. USA, 91:3515-19 (1994), and see also Coulie, et al., J. Exp. Med., 180:35-42 (1994).

An alternate approach toward characterization of CTL epitopes is to identify them directly. Naturally occurring  
25 peptides associated with MHC molecules on the tumor cells are directly extracted, fractionated by HPLC and used to reconstitute recognition by tumor specific CTL of a non-tumor cell expressing appropriate MHC molecules. Sequencing can be performed by Edman degradation. Mandelboim, et al., Nature,  
30 369:67-71 (1994) (CTL epitope on murine lung carcinoma). However, Applicants pioneered the use of tandem mass spectrometry to evaluate HHC-associated peptides. C.L. Slingluff, et al., J. Immunol. 150, 2955 (1993); D.F. Hunt, et al., Science 255, 1261 (1992); R.A. Henderson, et al.,  
35 Proc. Natl. Acad. Sci. USA 90, 10275 (1993).

However, when peptides associated with MHC molecules on tumor cells are extracted, a complex mixture, of up to 10,000-20,000 different peptides of similar size (mostly

nonamers), is obtained. Within this mixture, only a small number of molecules are likely to correspond to the peptides of interest. Consequently, their isolation and sequencing was extremely difficult. Boone, et al., Ann. Rev. Immunol., 5 12:337-65 (1994) states, "to our knowledge, the peptide elution method has not yet ensured the identification of a peptide recognized by anti-tumor CTL". More colorfully, Finn, et al., Curr. Op. Immunol., 5:701-8 (1993) likened the process to "throwing a fish hook into the ocean, hoping to 10 catch the big one", given, inter alia, the "very low amounts of peptides".

In the present invention, HLA associated peptides have been extracted, isolated and identified from different melanoma lines. These peptides can be used to reconstitute 15 epitopes for HLA-A2.1- restricted melanoma-specific CTL. These peptides and the stimulated CTL may be useful for the in vivo immunotherapeutic treatment of melanoma. Aspects of applicants' invention were described in Cox, et al., Science, 264:716-719 (1994), which was published on April 29, 1994.

### SUMMARY OF THE INVENTION

The present invention relates to immunogens which are capable of eliciting a melanoma-specific cytotoxic lymphocyte response in at least some individuals, which response is  
5 directed to peptide epitopes carried by those immunogens, and to the use of those immunogens in active specific immunotherapy and immunoprophylaxis against melanoma.

These immunogens may be used as vaccines, in active specific immunotherapy. The immunogens may be administered  
10 directly or by gene therapy. The epitopic peptides may also be used to stimulate lymphocytes, the latter then being used for adoptive immunotherapy. Peptides 946I and 946L, related to a single segment in pMel-17 (a protein homologous to gp100), had unexpectedly high CTL stimulatory activity. They  
15 also are recognized by CTL from different individuals.

Applicants used a peptide elution strategy to identify CTL epitopes among oligopeptides corresponding to selected segments of pMel-17. The use of a novel apparatus, which split a microcapillary stream into two streams in capillaries  
20 of different diameter and thereby permitted simultaneous immunological and mass spectrometric analysis of the effluent from a microcapillary HPLC column used for the initial fractionation, greatly facilitated this evaluation. For example, an HPLC fraction which mass spectra showed to  
25 contain at least 25 peptides contained only three whose relative abundances matched the activity profile for CTLs from DM 204. One of these three (peptide 946) was later confirmed as active. It was not predictable, prior to this work, that this splitting could be achieved without undue  
30 turbulence. Additional embodiments of the present invention are described below.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a graph of the specificity of VMM5 CTL on day 39;

Figure 2 is a graph illustrating the specificity of VMM5 CTL against a panel of 13 targets;

Figure 3 is a graph of the reconstitution of HLA-A2.1-restricted melanoma-specific epitopes for CTL using peptides eluted from A2.1 molecules on DM93;

Figure 4 is a graph of the reconstitution of melanoma-specific epitopes using reversed phase HPLC fractionated peptide extracted from HLA-A2.1 molecules expressed on DM6 cells;

Figure 5 illustrates in a graph the reconstitution of melanoma-specific epitopes using reversed phase HPLC-fractionated peptide extracted from HLA-A2.1 molecules expressed on DM93 cells;

Figure 6 illustrates graphically the titration of peptide dose for reconstitution;

Figure 7 graphs the second dimension separations of peptide extracts;

Figure 8 illustrates the reconstitution of epitopes for two melanoma-specific CTL lines with HPLC fractions derived from naturally processed peptides extracted from HLA-A2.1 molecules;

Figure 9 graphs the identification of candidate peptides by mass spectrometry;

Figure 10 illustrates the dose-titration curves for synthetic peptides;

Figure 11 illustrates the binding of synthetic 946 peptides to HLA-A2.1;

Figure 12 illustrates the peptide 946 reconstitutes epitope for 5 melanoma-specific CTL lines in a standard chromium release assay.

Figure 13 is a side view of the splitter; and

Figure 14 is a side view of peptide sequencing by microcapillary liquid chromatography/electrospray ionization tandem mass spectrometry.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS  
OF THE INVENTION**

The present invention relates to certain melanoma-specific CTL epitopes, and their incorporation into immunogens for immunoprophylactic and immunotherapeutic purposes. For the purpose of the present invention, a melanoma-specific CTL epitope is an epitope which is recognized by a T-cell receptor of at least some cytotoxic lymphocytes of at least some individuals in the population of interest, and which is more frequently or strongly associated with melanoma cells than with at least some other cancer and/or normal cells. There may be some cross-reactivity, for example, with other cells of melanocytic lineage. Absolute specificity is not required, provided that a useful prophylactic, therapeutic or diagnostic effect is still obtained.

**Melanoma-Specific CTL Epitopes**

The melanoma-specific CTL epitopes of the present invention are peptides, typically 9-11 amino acids in length, which are identical to or otherwise substantially homologous with melanoma-specific peptide epitopes recognized by melanoma-specific CTLs. The family of melanoma epitopes which are recoverable from an individual is dependent on the nature of the binding site of the Class I MHC (HLA) molecules expressed by the individual, and, as a result of the polymorphism of the Class I MHC (HLA) molecules, can vary considerably from one individual to another. For the purpose of the present invention, the melanoma cell line used as a source of melanoma-specific CTL epitopes may be any melanoma cell line; similarly, the Class I MHC (HLA) molecule may be any such molecule borne by a melanoma which is capable of binding to and presenting a melanoma-specific epitope, including, but not limited to, the various allelic forms of Class I MHC molecules enumerated in Table I. Epitopes presentable by HLA-A2.1 are of particular interest, as it is a common form of HLA-A. The preferred epitopic sequence may vary depending on the restriction system.

Preferably, the CTL epitopes of the present invention, in the cytotoxicity assay described hereafter, when used in oligopeptide form to stimulate suitable CTL, achieve, at the dosage resulting in maximal lysis of melanoma cells exposed to the stimulated CTL, a percentage lysis of melanoma cells which is at least 10 percentage points higher (more preferably, at least 20 points higher) the background level of lysis of the melanoma cells by the CTLs (i.e., in absence of the peptide).

Preferably, the peptide concentration at which the epitope-stimulated CTLs achieve half the maximal increase in lysis relative to background is no more than about 1 nM, more preferably no more than about 100 pM, still more preferably no more than about 10 pM. For the peptides 946L and 946I, half-maximal lysis of T2 cells is observed with concentrations of peptide in the pM range. In contrast, the MAGE-1 peptide EADPTGHSY had half-maximal lysis between 1 and 100 nM (prob about 10); while the tyrosinase peptides YMNGTMSQV and MLLAVLYCL reported by Boon induced half-maximal lysis (even with pre-treatment with MA2.1 antibody) at over 10 nM.

Preferably the epitope is recognized by CTLs from at least two different individuals, more preferably at least five different individuals.

More preferably, the CTL epitope satisfies two or more of the above desiderata.

The 946L peptide, although recognized by HLA-A2.1-restricted melanoma-specific CTL, may not be optimal at present. It is known that some residues on the nonamer peptide are particularly important for binding of the peptide to the MHC molecule (residues 2,9), while others are particularly important for Tc recognition (residues 4-8). The other residues may be important for either or both. It is proposed that amino acid substitutions for the 946 peptide may be useful at increasing immunogenicity, particularly by attempting to change residues that may increase binding to the MHC such as changing residue 9 to a valine or residue 3 to anything other than glutamic acid (E). Using existing knowledge about which of these residues may be more likely to

affect binding either to the MHC or to the TcR, a rational approach to this process may be employed. The resulting peptides, if more effective, could be used for any of the purposes described in this proposal. (refs: E.L. Huczko et al. J. Immunol. 151:2572, 1993; J. Ruppert et al. Cell 754: 929, 1993; Madden Dr et al. Cell 75:693-708, 1994.)

Therefore, in addition to epitopes which are identical to the naturally occurring melanoma-specific epitopes, the present invention embraces epitopes which are substantially homologous with such epitopes, and therefore melanoma-specific in their own right.

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence with each other, giving rise to a homology in conformation and thus to similar (or improved) biological activity. The term is not intended to imply a common evolution of the sequences.

Substantially homologous peptide epitopes may be identified by a variety of techniques. It is known in the art that one may synthesize all possible single substitution mutants of a known peptide epitope. For a nonpeptide, there are  $(20 \times 9 - 1 = 179)$  such mutants. Geysen, et al., Proc Nat. Acad. Sci. (USA), 81:3998-4002 (1984). While the effects of different substitutions are not always additive, it is reasonable to expect that two favorable or neutral single substitutions at different residue positions in the epitope can safely be combined in most cases.

One may also synthesize a family of related single or multiple substitution mutants, present the mixture to the HLA-A2.1 positive lymphoblastoid cell line T2 (or other cell line capable of presenting melanoma-specific CTL epitopes), and expose the T2 cells to melanoma-specific CTLs. If the T2 cells are lysed, the effective epitopes may be identified either by direct recovery from the T2 cells or by a progressive process of testing subsets of the effective peptide mixtures. Methods for the preparation of degenerate peptides are described in Rutter, USP 5,010,175, Haughten, et

al., Proc. Nat. Acad. Sci. (USA), 82:5131-35 (1985), Geysen, et al., Proc. Nat. Acad. Sci. (USA), 81:3998-4002 (1984); W086/06487; W086/00991.

In the case of the peptide 946L (SEQ. ID. No.:14), a possible multiple mutagenesis strategy would be as follows:

	<u>Parental</u>	<u>Tyr</u>	<u>Leu</u>	<u>Glu</u>	<u>Pro</u>	<u>Gly</u>	<u>Pro</u>	<u>Val</u>	<u>Thr</u>	<u>Ala</u>
	Possible	Phe	Ile	Asp	Ala	Pro	Ala	Ile	Ala	Thr
	Mutations	Trp	Val		Ser	Ala	Ser	Leu	Ser	Ser
			Met		Thr	Ser	Thr	Met	Pro	Pro
10					Gly	Thr	Gly		Gly	Gly

Assuming that the "parental" amino acid is allowed to occur at each position, there would be about 300,000 different peptides in this family.

For peptide 1030 (SEQ. ID NO.:9), a possible strategy would be:

	<u>Parental</u>	<u>Tyr</u>	<u>Met</u>	<u>Asp</u>	<u>Gly</u>	<u>Thr</u>	<u>Met</u>	<u>Ser</u>	<u>Gln</u>	<u>Val</u>
		Phe	Val	Glu	Pro	Ala	Val	Ala	Asn	Ile
		Try	Ile		Ala	Ser	Ile	Thr		Leu
			Leu		Ser	Pro	Leu	Pro		Met
20					Thr	Gly		Gly		

This family has 96, 0000 members. Other strategies are, of course, possible. For example, the Asp/Glu and Gln/Asn sets can be merged. It is known from comparison of peptide 1030 with the homologous tyrosinase segment that substitution of Asn for Asp in position 3 reduces CTL activity 100-fold. However, a multiple mutagenesis strategy could identify compensating mutations at other sites.

The person of ordinary skill in the art, in determining which residues to vary, may also make comparisons of the sequences of the naturally processed MHC associated peptides, and may obtain 3D structures of the MHC: peptide: TCR complexes, in order to identify residues involved in MHC or TCR binding. Such residues may either be left alone, or judiciously mutated in an attempt to enhance MHC or TCR binding.

It is also possible to predict substantially homologous epitopes by taken into account studies of sequence variations in families of naturally occurring homologous proteins. Certain amino acid substitutions are more often tolerated

than others, and these are often correlatable with similarities in size, charge, etc. between the original amino acid and its replacement. Insertions or deletions of amino acids may also be made.

5       Conservative substitutions are herein defined as exchanges within one of the following five groups:

I.    Small aliphatic, nonpolar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly

10    II. Polar, negatively charged residues: and their amides

Asp, Asn, Glu, Gln

III. Polar, positively charged residues:

His, Arg, Lys

15    IV. Large, aliphatic, nonpolar residues:

Met, Leu, Ile, Val, Cys

V.    Large, aromatic residues:

Phe, Tyr, Trp

20       Within the foregoing groups, the following substitutions are considered "highly conservative":

Asp/Glu

His/Arg/Lys

Phe/Tyr/Trp

Met/Leu/Ile/Val

25       Semi-conservative substitutions are defined to be exchanges between two of groups (I)-(V) above which are limited to supergroup (A), comprising (I), (II) and (III) above, or to supergroup (B), comprising (IV) and (V) above.

30       Substitutions are not limited to the genetically encoded, or even the naturally occurring amino acids. When the epitope is prepared by peptide synthesis, the desired amino acid may be used directly. Alternatively, a genetically encoded amino acid may be modified by reacting it with an organic derivatizing agent that is capable of reacting with  
35       selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

Aromatic amino acids may be replaced with D- or L-naphylalanine, D- or L-Phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or  
5 L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole-  
10 (alkyl)alanines, and D- or L-alkylalanines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, iso-propyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-  
15 carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)-alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g.,  $-SO_3H$ ) threonine, serine, tyrosine.

20 Other substitutions may include unnatural hydroxylated amino acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine,  
25 ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is defined as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for  
30 methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amide linkage can be replaced by a ketomethylene moiety, e.g.  $(-C(=O)-CH_2-)$  for  $(-(C=O)-NH-)$ . Such derivatives are expected to have the property of  
35 increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased in vivo half lives, as administered by oral,

intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

In addition, any amino acid can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S configuration, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D-amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability to degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer in vivo half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications of amino acids may include the following: Cysteiny l residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysiny l and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to

have the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues per se is well-known, such as for introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be readily deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to a water-insoluble support matrix or to other macromolecular carriers, according to known

method steps. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of the C-terminal carboxyl groups, according to known method steps. Glycosylation is also possible.

Derivatized moieties may improve the solubility, absorption, biological half life, and the like, or eliminate or attenuate any possible undesirable side effect of the molecule. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980).

#### The Melanoma-Specific Immunogen

The melanoma-specific immunogen of the present invention is a molecule corresponding to or otherwise comprising a melanoma-specific CTL epitope as previously described. The

immunogen may comprise one or more melanoma-specific CTL epitopes, which may be the same or different. If different, the epitopes may be chosen so that at least one epitope is effective in each of two or more restriction systems, e.g., HLA-A1 and HLA-A2. If the immunogen comprises a plurality of such epitopes, they may be linked directly, or through a spacer of some kind, or by noncovalent means such as an avidin:biotin complex. The immunogen may take any form that is capable of eliciting a melanoma-specific cytotoxic immune response. By way of example and not of limitation, the immunogen may be a fusion of a plurality of CTL epitopes which is sufficiently large to be immunogenic, a conjugate of one or more epitopes to a soluble immunogenic macromolecular carrier, such as serum albumin, keyhole limpet hemocyanin, or dextran, a recombinant virus engineered to display the epitope on its surface, or a conjugate of a plurality of epitopes to a branched lysine core structure, a so-called "multiple antigenic peptide" (see Posnett, et al., J. Biol. Chem., 263:1719-25, 1988). The immunogenic conjugate may also comprise moieties intended to enhance the immune response, such as a cytokine or an adjuvant.

#### Mode of Production

The peptide portion of the immunogens of the present invention may be produced by any conventional technique, including

- (a) nonbiological synthesis by sequential coupling of component amino acids,
- (b) production by recombinant DNA techniques in a suitable host cell, and
- (c) chemical or enzymatic modification of a sequence made by (a) or (b) above.

*Gene Expression.* The peptides disclosed herein may be produced, recombinantly, in a suitable host, such as bacteria from the genera *Bacillus*, *Escherichia*, *Salmonella*, *Erwinia*, and yeasts from the genera *Hansenula*, *Kluyveromyces*, *Pichia*, *Rhinosporidium*, *Saccharomyces*, and *Schizosaccharomyces*, or

cultured mammalian cells such as COS-1. The more preferred hosts are microorganisms of the species *Pichia pastoris*, *Bacillus subtilis*, *Bacillus brevis*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica*. Any promoter, 5 regulatable or constitutive, which is functional in the host may be used to control gene expression.

It has been found that a peptide fragment from the protein pMEL17 reconstitutes an HLA epitope. The pMEL17 gene is a single-stranded cDNA reading 5' to 3'. The gene 10 encoding for pMEL17, SEQ. ID. NO. 91 is:

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GGAAGAACAC AATGGATCTG GTGCTAAAAA GATGCCTTCT TCATTGCGCT
GTGATAGGTG CTTTGCTGGC TGTGGGGGCT ACAAAGTAC CCAGAAACCA
GGACTGGCTT GGTGTCTCAA GGCAACTCAG AACCAAAGCC TGGAACAGGC
AGCTGTATCC AGAGTGGACA GAAGCCCAGA GACTTGACTG CTGGAGAGGT
15 GGTCAAGTGT CCCTCAAGGT CAGTAATGAT GGGCCTACAC TGATTGGTGC
AAATGCCTCC TTCTCTATTG CCTTGAACCT CCCTGGAAGC CAAAAGGTAT
TGCCAGATGG GCAGGTTATC TGGGTCAACA ATACCATCAT CAATGGGAGC
CAGGTGTGGG GAGGACAGCC AGTGTATCCC CAGGAACTG ACGATGCCTG
CATCTTCCCT GATGGTGGAC CTGCCCATC TGGCTCTTGG TCTCAGAAGA
20 GAAGCTTTGT TTATGTCTGG AAGACCTGGG GCCAATACTG GCAAGTTCTA
GGGGGCCAG TGTCTGGGCT GAGCATTGGG ACAGGCAGGG CAATGCTGGG
CACACACACC ATGGAAGTGA CTGTCTACCA TCGCCGGGGA TCCCGGAGCT
ATGTGCCTCT TGCTCATTC AGCTCAGCCT TCACCATTAC TGACCAGGTG
CCTTTCTCCG TGAGCGTGTC CCAGTTGCGG GCCTTGATG GAGGGAACAA
25 GCACTTCCTG AGAAATCAGC CTCTGACCTT TGCCCTCCAG CTCCATGACC
CTAGTGGCTA TCTGGCTGAA GCTGACCTCT CCTACACCTG GGACTTTTGA
GACAGTAGTG GAACCCTGAT CTCTCGGGCA CCTGTGGTCA CTCATACTTA
CCTGGAGCCT GGCCAGTCA CTGCCCAGGT GGTCCTGCAG GCTGCCATTG
CTCTCACCTC CTGTGGCTCC TCCCAGTTC CAGGCACCAC AGATGGGCAC
30 AGGCCAACTG CAGAGGCCCC TAACACCACA GCTGGCCAAG TGCCTACTAC
AGAAGTTGTG GGTACTACAC CTGGTCAGGC GCCAACTGCA GAGCCCTCTG
GAACCACATC TGTGCAGGTG CCAACCACTG AAGTCATAAG CACTGCACCT
GTGCAGATGC CAACTGCAGA GAGCACAGGT ATGACACCTG AGAAGGTGCC
AGTTTCAGAG GTCATGGGTA CCACACTGGC AGAGATGTCA ACTCCAGAGG
35 CTACAGGTAT GACACCTGCA GAGGTATCAA TTGTGGTGCT TTCTGGAACC
ACAGCTGCAC AGGTAACAAC TACAGAGTGG GTGGAGACCA CAGCTAGAGA
GCTACCTATC CCTGAGCCTG AAGGTCCAGA TGCCAGCTCA ATCATGTCTA
CGGAAAGTAT TACAGGTTCC CTGGGCCCCC TGCTGGATGG TACAGCCACC
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TTAAGGCTGG TGAAGAGACA AGTCCCCCTG GATTGTGTTC TGTATCGATA  
TGGTTCCTTT TCCGTCACCC TGGACATTGT CCAGGGTATT GAAAGTGCCC  
AGATCCTGCA GGCTGTGCCG TCCGGTGAGG GGGATGCATT TGAGCTGACT  
GTGTCCTGCC AAGGCGGGCT GCCCAAGGAA GCCTGCATGG AGATCTCATC  
5 GCCAGGGTGC CAGCCCCCTG CCCAGCGGCT GTGCCAGCCT GTGCTACCCA  
GCCCAGCCTG CCAGCTGGTT CTGCACCAGA TACTGAAGGG TGGCTCGGGG  
ACATACTGCC TCAATGTGTC TCTGGCTGAT ACCAACAGCC TGGCAGTGGT  
CAGCACCCAG CTTATCATGC CTGTGCCTGG GATTCTTCTC ACAGGTCAAG  
AAGCAGGCCT TGGGCAGGTT CGGCTGATCG TGGGCATCTT GCTGGTGTG  
10 ATGGCTGTGG TCCTTGATC TCTGATATAT AGGCGCAGAC TTATGAAGCA  
AGACTTCTCC GTACCCCACT TGCCACATAG CAGCAGTCAC TGGCTGCGTC  
TACCCCGCAT CTTCTGCTCT TGTCCCATTG GTGAGAATAG CCCCCTCCTC  
AGTGGGCAGC AGGTCTGAGT ACTCTCATAT GATGCTGTGA TTTTCTGGA  
GTTGACAGAA ACACCTATAT TTCCCCCAGT CTTCCCTGGG AGACTACTAT  
15 TAACTGAAAT AAATACTCAG AGCCTGAAAA A

Of particular importance within the context of the present invention is the peptide 946L YLEPGPVTA (SEQ. ID NO:14) and its encoding gene sequence, (SEQ. ID. NO. 90) TAC CTG GAG CCT GGC CAA GTC ACT GCC. Because this peptide has  
20 proven immunologic activity, it is ideal for specific immunization. Such immunization may be accomplished either directly, or by use of a vaccine consisting of virus (e.g., Vaccinia) encoding or HLA-A2 cells expressing a genetic sequence encoding this peptide. Also promising is the gene  
25 sequence encoding peptide 1030, YMDGTMSQV (SEQ. ID NO.:9) TAT ATG GAT GGA ACA ATG TCC GAG GTA (SEQ. ID. NO.:92).

These sequences may be constructed in such a manner, including the appropriate expression systems for use in gene therapy procedures. Because several different nucleotide  
30 sequences may encode a single amino acid, alternate DNA sequences may also encode these peptides.

Standard reference works setting forth the general principles of recombinant DNA technology include Watson, J.D., et al., Molecular Biology of the Gene, Volumes I and  
35 II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, J.E., et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II,

John Wiley & Sons, publishers, New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981);

5 Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Ausubel, et al., Current Protocols in Molecular Biology, Wiley Interscience, N.Y., (1987, 1992). These references are herein entirely incorporated by reference.

10 Chemical Peptide Synthesis. Chemical peptide synthesis is a rapidly evolving area in the art, and methods of solid phase peptide synthesis are well-described in the following references, hereby entirely incorporated by reference:

(Merrifield, B., J. Amer. Chem. Soc. 85:2149-2154 (1963);

15 Merrifield, B., Science 232:341-347 (1986); Wade, J.D., et al., Biopolymers 25:S21-S37 (1986); Fields, G.B., Int. J. Polypeptide Prot. Res. 35:161 (1990); MilliGen Report Nos. 2 and 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel, et al, supra, and Sambrook, et al, supra.

20 In general, as is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed (or de-protected).

25 Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particle large enough to be separated from the fluid phase by filtration. Thus, reactants are removed by washing

30 the resin particles with appropriate solvents using an automated programmed machine. The completed polypeptide chain is cleaved from the resin by a reaction which does not affect polypeptide bonds.

In the more classical method, known as the "tBoc method," the amino group of the amino acid being added to the

35 resin-bound C-terminal amino acid is blocked with tert-butyloxycarbonyl chloride (tBoc). This protected amino acid is reacted with the bound amino acid in the presence of

the condensing agent dicyclohexylcarbodiimide, allowing its carboxyl group to form a polypeptide bond the free amino group of the bound amino acid. The amino-blocking group is then removed by acidification with trifluoroacetic acid (TFA); it subsequently decomposes into gaseous carbon dioxide and isobutylene. These steps are repeated cyclically for each additional amino acid residue. A more vigorous treatment with hydrogen fluoride (HF) or trifluoromethanesulfonyl derivatives is common at the end of the synthesis to cleave the benzyl-derived side chain protecting groups and the polypeptide-resin bond.

More recently, the preferred "Fmoc" technique has been introduced as an alternative synthetic approach, offering milder reaction conditions, simpler activation procedures and compatibility with continuous flow techniques. This method was used, e.g., to prepare the peptide sequences disclosed in the present application. Here, the  $\alpha$ -amino group is protected by the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group. The benzyl side chain protecting groups are replaced by the more acid labile t-butyl derivatives. Repetitive acid treatments are replaced by deprotection with mild base solutions, e.g., 20% piperidine in dimethylformamide (DMF), and the final HF cleavage treatment is eliminated. A TFA solution is used instead to cleave side chain protecting groups and the peptide resin linkage simultaneously.

At least three different peptide-resin linkage agents can be used: substituted benzyl alcohol derivatives that can be cleaved with 95% TFA to produce a peptide acid, methanolic ammonia to produce a peptide amide, or 1% TFA to produce a protected peptide which can then be used in fragment condensation procedures, as described by Atherton, E., et al., J. Chem. Soc. Perkin Trans. 1:538-546 (1981) and Sheppard, R.C., et al., Int. J. Polypeptide Prot. Res. 20:451-454 (1982). Furthermore, highly reactive Fmoc amino acids are available as pentafluorophenyl esters or dihydro-oxobenzotriazine esters derivatives, saving the step of activation used in the tBoc method.

Pharmaceutical Methods and Preparations

The preferred animal subject of the present invention is a primate mammal. By the term "mammal" is meant an individual belonging to the class Mammalia, which, of course, includes humans. The invention is particularly useful in the treatment of human subjects, although it is intended for veterinary uses as well. By the term "non-human primate" is intended any member of the suborder Anthroidea except for the family Hominidae. Such non-human primates include the superfamily Ceboidea, family Cebidae (the New World monkeys including the capuchins, howlers, spider monkeys and squirrel monkeys) and family Callithricidae (including the marmosets); the superfamily Cercopithecoidea, family Cercopithecidae (including the macaques, mandrills, baboons, proboscis monkeys, mona monkeys, and the sacred hunaman monkeys of India); and superfamily Hominoidea, family Pongidae (including gibbons, orangutans, gorillas, and chimpanzees). The rhesus monkey is one member of the macaques.

The term "protection", as used herein, is intended to include "prevention," "suppression" and "treatment." "Prevention" involves administration of the protein prior to the induction of the disease. "Suppression" involves administration of the composition prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after the appearance of the disease.

It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or events. Therefore, it is common to use the term "prophylaxis" as distinct from "treatment" to encompass both "preventing" and "suppressing" as defined herein. The term "protection," as used herein, is meant to include "prophylaxis." It should also be understood that to be useful, the protection provided need not be absolute, provided that it is sufficient to carry clinical value. An

agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the level of protection, or if it is safer than competitive agents.

The form of administration may be systemic or topical. For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

A typical regimen comprises administration of an effective amount of the immunogen, administered over a period ranging from a single dose, to dosing over a period of hours, days, weeks, months, or years.

It is understood that the suitable dosage of a immunogen of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This will typically involve adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

Prior to use in humans, a drug will first be evaluated for safety and efficacy in laboratory animals. In human clinical studies, one would begin with a dose expected to be safe in humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs (if any). If this dose is effective, the dosage may be decreased, to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients monitored for signs of side effects. See, e.g., Berkow, et al., eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman, et

al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, Pharmacology, Little, Brown and Co., Boston, (1985), which references and references cited therein, are entirely incorporated herein by reference.

The total dose required for each treatment may be administered by multiple doses or in a single dose. The immunogen may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

The appropriate dosage form will depend on the disease, the immunogen, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories, inhalants, solutions, ointments and parenteral depots. See, e.g., Berker, supra, Goodman, supra, Avery, supra and Ebadi, supra, which are entirely incorporated herein by reference, including all references cited therein. However, it is expected that each vaccine preparation will include 1-100  $\mu$ g of the peptide epitope.

In addition to at least one immunogen as described herein, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. See, e.g., Berker, supra, Goodman, supra, Avery, supra and Ebadi, supra, which are entirely incorporated herein by reference.

The composition may also include an adjuvant. Typical adjuvants include proteins, peptides, carbohydrates, lipids and liposaccharides. An example of a currently popular adjuvant is DETOX (Ribi Immunochemicals) (muramyl dipeptide and cell wall fragments from Mycobacterium phlei). If desired, the adjuvant may be conjugated to the epitope and not simply a part of a mixture. See Deres, et al, Nature, 342:561-4 (1989).

The composition may also include an immunomodulator, especially cytokines such as IL-1, IL-2, IL-4, IL-6, IL-7, IL-12, Interferon-alpha, Interferon-gamma, Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Tumor Necrosis  
5 Factor (TNF)-alpha, and TNF- beta.

A pharmaceutical composition according to the present invention may further comprise at least one cancer chemotherapeutic compound, such as one selected from the group consisting of an anti-metabolite, a bleomycin peptide  
10 antibiotic, a podophyllin alkaloid, a Vinca alkaloid, an alkylating agent, an antibiotic, cisplatin, or a nitrosourea. A pharmaceutical composition according to the present invention may further or additionally comprise at least one  
15 viral chemotherapeutic compound selected from gamma globulin, amantadine, guanidine, hydroxybenzimidazole, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , thiosemicarbazones, methisazone, rifampin, ribvirin, a pyrimidine analog, a purine analog, foscarnet, phosphonoacetic acid, acyclovir, dideoxy-  
nucleosides, or ganciclovir. See, e.g., Katzung, *supra*, and  
20 the references cited therein on pages 798-800 and 680-681, respectively, which references are herein entirely incorporated by reference.

As an alternative to a pharmaceutical composition comprising the immunogen of the present invention, per se,  
25 the pharmaceutical composition may instead comprise a vector comprising an expressible gene encoding such an immunogen. The pharmaceutical composition and method would then be chosen so that the vector was delivered to suitable cells of the subject, so that the gene would be expressed and the  
30 immunogen produced in such a manner as to elicit an immune response. A preferred vector would be a Vaccinia virus, such as a construct containing a minigene encoding the peptide 946L (YLEPGPVTA) or 946I ((YIEPGPVTA), or 1030 (SEQ. ID. NO.: 9). A gene encoding the protein pMel-17 is also of some  
35 interest. In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature. A preferred route for immunization would

be scarification. A preferred immunization protocol would be 10E6 to 10E8 pfu/dose in the initial injection, followed up with boosters at 1,3 and 12 months. The boosters could be the previously described immunogen-containing composition.

5 In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature.

Recombinant vaccinia virus constructs have been used for  
10 immunization against hepatitis B (Moss, et al., Nature, 311, 67, 1984), herpes simplex virus (Wacchsman, et al., Biosci. Rep. 8, 323; 334, 1988), parainfluenza type 3 (Spriggs, et al., J. Virol., 62, 1293, 1988), and Lassa fever virus (Fisher-Hoch, et al., Proc. Natl. Acad. Sci. USA, 86, 317,  
15 1989). Vaccinia virus constructs comprising gene for cancer-associated antigens have also been prepared (Lathe, et al., Nature, 326, 878, 1987; Bernards, et al., Proc. Natl. Acad. Sci. USA, 84, 6854, 1987; Estin, et al., Proc. Natl. Acad. Sci. USA, 85, 1052, 1988).

20 Adoptive transfer of melanoma-specific CTL has been accompanied by tumor shrinkage in a large minority of patients with advanced melanoma and by disappearance of all detectable tumor in a smaller proportion of patients. (Rosenberg et al, NEM 319: 1676-1680, 19888) and in animal  
25 studies appears to be particularly promising for the treatment of solid tumors (Rosenberg SA et al. Science 233:1318-1321). One of the problems with existing methods for CTL generation is that they require the resection of large metastatic tumor deposits to initiate the process. If the  
30 requirement for available autologous tumor could be circumvented, then patients with no measurable disease but a high risk of recurrence (eg, melanoma patients with primary tumors greater than 4 mm thick or with tumor metastatic to regional nodes) could be treated with adoptive therapy even  
35 if their tumor were removed and fixed in formalin and no other gross tumor was evident. These patients have a very high likelihood of harboring micrometastatic disease for which no other effective therapy is now available; so most will die

of the melanoma. It is possible that the presence of bulky tumor suppresses the autologous immune response; so treatment of patients without bulky disease would be an attractive goal. Especially in murine systems, CTL have been generated and maintained by stimulation with cells to which the peptide epitope has been bound. We propose that HLA-A2.1+ cells (autologous B cells, macrophages, or dendritic cells, ideally) would be pulsed in vitro with peptide (e.g., peptide 946, YXEPGPVTA) and used as in vitro simulators for autologous lymph node cells or peripheral blood lymphocytes. The patients could be pre-stimulated with a peptide vaccine prior lymphocyte harvest if the existing response was inadequate. Lymphocytes stimulated with peptide in vitro could then be expanded to  $10^{10}$  or  $10^{11}$  cells, then re-infused into the patients in a manner analogous to that used for LAK cell therapy. It is expected that the adoptively transferred CTL would survive best with IL-2 infusion at low to intermediate doses, and that putative inhibitors of Tc suppression (eg: cyclophosphamide) may be employed also, prior to the infusions of CTL.

#### Nontherapeutic Uses and Compositions

The relationship between the host's immune response and his or her tumor is poorly understood. Better understanding of that response depends on evaluation of the specific responses against individual epitopes, such as the 946 peptide. If patients do have an immune response to 946 naturally, then evaluation and quantitation of that by precursor frequency analysis of the CTL in the patient's blood pool may permit some assessment of the protection that person's immune system is providing. As new therapies become available for melanoma, it may be useful to screen patients for the presence of the 946 peptide on their tumor and the presence of CTL in their blood pool with specificity for the 946 peptide on HLA-A2. These findings may determine whether further augmentation of the immune response is indicated or whether other, non-immunologic, therapy should be employed. A parallel to this is the determination on breast cancers of

the presence of estrogen and progesterone receptors before considering hormonal therapy or chemotherapy.

Thus, the peptides of the present invention may be used to screen a sample for the presence of an antigen with the same epitope, or with a different but cross-reactive epitope, or for the presence of CTLs which specifically recognize the corresponding epitopes. The sample will normally be a biological fluid, such as blood, urine, lymphatic fluid, amniotic fluid, semen, saliva, tears, milk, or cerebrospinal fluid, or a fraction or derivative thereof, or a biological tissue, in the form of, e.g., a tissue section or homogenate. The preferred sample is blood, or a fraction or derivative thereof.

Assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

Assays may also be divided into competitive and non-competitive formats. In the competitive format, the analyte competes with a labeled analyte analogue for binding to a binding partner. In a common noncompetitive format called a sandwich assay, the analyte is first bound by a capture reagent, and then by a tag reagent.

In order to detect the presence, or measure the amount, of an analyte, the assay must provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only with instruments.

Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation

or agglutination of a component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , and, preferably,  $^{125}\text{I}$ .

It is also possible to label a compound with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as  $^{125}\text{Eu}$ , or others of the lanthanide series, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) and ethylenediamine-tetraacetic acid (EDTA).

The peptides also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the peptides. Bioluminescence is a type of chemiluminescence

found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bio-  
5 luminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase, alkaline phosphatase, malate dehydrogenase, staphylococcal nuclease,  $\delta$ -V-steroid isomerase, yeast alcohol dehydrogenase,  $\alpha$ -glycero  
10 phosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase,  $\beta$ -galactosidase, ribonuclease, glucose-6-phosphate dehydrogenase, glucoamylase and acetyl-  
choline esterase, are preferred. When an enzyme label is used, the signal producing system must also include a  
15 substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

A label may be conjugated, directly or indirectly (e.g., through a labeled antibody), covalently (e.g., with SPDP) or  
20 noncovalently, to the peptide, to produce a diagnostic reagent. Similarly, the peptide may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases,  
25 natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled  
30 molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

35 Additionally, the peptides may be used as a diagnostic tool to evaluate whether other immunotherapeutic treatments (tumor vaccines of any kind, adoptive transfer of CTL, etc) are having a beneficial effect.

Also the peptides 946L (YLEPGPVTA) and 946I (YIEPGPVTA) have very low affinity for the HLA-A2.1 molecule. This is illustrated in Figure 11. For this reason, they will be useful as control peptides for the evaluation of candidate peptide/MHC binding affinity. Because they represent a low affinity range, they can be used in laboratory studies on binding affinity of other peptides. This methodology, in a preferred embodiment, would likely include: binding the peptide to T2 cells, then evaluating lysis of the T2 cells by any of various standard methods, such as a proliferative response of the CTL, or cytokine release by the CTL exposed to the T2 cells+ peptide.

**EXAMPLES****Materials and Methods - Cell lines and HLA typing**

All tumor cell lines were of human origin. Melanoma cell lines HT144 and Sk-Mel-24, osteosarcoma 143b, colon cancer CCL- 228 (SW480), and breast cancer MDA-MB-468 were obtained from the American Type Culture Collection (Bethesda, MD). Fibroblasts GM126 were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Bethesda, MD. Melanoma lines DM6, DM13, DM14, and DM93 were the gift of Drs. Hilliard F. Siegler and Timothy L. Darrow. VMM1 and VMM5 are melanoma cell lines established from metastatic melanoma resected from patients at the University of Virginia (Charlottesville, VA). VBT2 (squamous cell lung carcinoma), VA01 (adenocarcinoma of the ovary), and VAB5 (adenocarcinoma of the breast) are cell lines also established at this institution. JY, MICH, MWF, 23.1, RPMI 1788, and Herluff are EBV-transformed B lymphoblastoid lines. K562 is a NK-sensitive human erythroleukemia line. The cell line T2 is derived from the fusion of a T cell line, CEM, and a human B cell mutant, LCL 721.174. This cell line expresses HLA-A2.1 molecules but has an Ag-processing defect that is associated with enhanced presentation of exogenous peptides.

HLA types of the specimens used in this study are summarized in Table 1.

TABLE 1  
Human cell lines used in this study: HLA types and susceptibility to lysis by VM5 CTL<sup>a</sup>

Cell Line (Ref.)	Cell Type <sup>b</sup>	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	Lysis by VM5 CTL <sup>c</sup>
DM6 (11)	Melanoma	2.1 <sup>e</sup>	12, 13 or 35	1, 2	6.10.(7) <sup>d</sup>	---	++
DM13 5(11)	Melanoma	2.1, 31	13, 18	ND	ND	ND	+++
DM14 (11)	Melanoma	11, 28	5, 8	2, 4	---	---	---
DM93 (11)	Melanoma	2.1, 33	8, 49, w6	ND	2, 4, 6 <sup>d</sup>	3	++
SKMel24 (26)	Melanoma	1, 2.1	12, 14	---	---	---	---
HT144 (26)	Melanoma	1, 24	13, 15	3	4, 7	---	---
HT144 A2--03	Melanoma	1, 2.1, 24	13, 15	3	4, 7	---	+
VM1	Melanoma	3, 26	51, w4, w6	ND	---	---	---
VM5	Melanoma	2.1	39	ND	7, 11, 52, 53	2, 7	+++
VBT2	Lung CA	34, 68	35, (53?)	4?	---	---	---
VAO1	Ovarian CA	2	---	---	---	---	---
VAB4 5	Breast CA	2, 25	60, 62	3	---	---	---
MDAMB468 (26)	Breast CA	23, 30	27, 35	2, 4	---	---	---
CCL228 (26)	Colon CA	2.1	8, 17	---	---	---	---
143b (29)	Osteosarcoma	2.1	---	---	---	---	---
GM126 (29)	Fibroblasts	2.1	---	---	---	---	---
K5620	Erythroleukemia	---	---	---	---	---	---
MICH (28)	EBV- $\delta^0$	2.1, 32	15, 27	---	5, 5	---	---
RPMI-1788 (26)	EBV- $\delta$	2.1, 33	7, 14	---	---	---	---
JY (28)	EBV- $\delta$	2.1, 2.1	7, 7	---	4, 6	---	---
Herluff (27)	EBV- $\delta$	2.1, 2.1	12, 35	---	---	---	---
23.25(28)	EBV- $\delta$	2, 2	27, 27	---	8, 8	---	---

Expression of HLA-A2 on tumor cells was assessed by staining with BB7.2. Expression of the A2 subtype HLA-A2.1 was confirmed by susceptibility to lysis by HLA-A2.1-specific murine CTL clones AT1-15 and AX21-9, and by staining with  
5 HLA-A2.1/A2.2-specific mAb CR11.351.

#### Generation of tumor-specific cytotoxic T cells

Detailed methods of CTL generation have been previously reported. Malignant melanoma metastatic to cervical lymph nodes was resected from an 80-yr old patient designated VMM5.  
10 The nodes were mechanically dissociated and then enzymatically digested in Eagle's MEM (GIBCO, Grand Island, NY) containing 2.5% FCS (GIBCO, or Whittaker, Walkersville, MD), 0.1% collagenase B (Boehringer-Mannheim, Indianapolis, IN), 0.002% DNase (Sigma, St. Louis, MO), penicillin 100  
15 U/ml, streptomycin 100 microg/ml (Pen- Strept, GIBCO) at room temperature. After 4 h, dissociated cells were collected and cryopreserved. Remaining tumor fragments were returned to the digestion media overnight. The digests were harvested in a similar fashion daily for 3 days, with viable tumor cells  
20 and lymphocytes obtained each day. These cells were cryopreserved in FCS + 10% DMSO in liquid nitrogen. Initial cultures were established with the mixture of lymphocytes and tumor from the tumor-involved node. The ratio of tumor cells to lymphocytes placed in culture were approximately 1:1. The  
25 cells were cultured in 24-well tissue culture plates (Linbro, Hamden, CT) in RPMI 1640 (Sigma) containing 10% FCS, Pen-Strept, and 20 U/ml rIL-2 (Cetus, Emeryville, CA). The CTL were restimulated with irradiated (100 Gy) fresh cryo-  
preserved autologous tumor (VMM5) at a TLR (tumor:lymphocyte  
30 ratio) of 1:10 on day 16. Beginning with the third in vitro stimulation (day 32), and thereafter every 10 to 15 days, the CTL were restimulated with the allogeneic HLA-A2.1<sup>+</sup> melanoma cell line DM6. A TLR of 1:5 was used until the cells were 70 days old, after which a TLR between 1:2 and 2:1 was used.  
35 Several VMM5 CTL lines were generated following this protocol closely and with consistent results from each. Similar

methods were employed for generation of other CTL lines studied.

#### Cytotoxicity Assays

Cell-mediated killing was determined in vitro using a 4-h chromium release assay. <sup>51</sup>Cr-labeled target cells were plated at 2 x 10<sup>3</sup> cells/well in triplicate on 96-well V-bottom plates (Costar, Cambridge, MA) with varying numbers of effector cells in a final volume of 250 microl. Wells containing either culture medium and target cells only or 1 M HCl and target cells served as background <sup>51</sup>Cr release controls and total release controls, respectively. The plates were centrifuged at 100 x g for 3 min and incubated at 37°C in 5% CO<sub>2</sub> for 4 h. The plates were again centrifuged, and 0.20 ml of medium from each well was removed for counting in a gamma counter. The cytotoxic index was calculated as:

$$\frac{\text{Cpm (experimental)} - \text{cpm (background)}}{\text{Cpm (total release)} - \text{cpm (background)}} \times 100\%$$

Lytic units were calculated for several of the cytotoxicity assays, using a software package prepared by the National Cancer Institute (Bethesda, MD), which solves for the equation  $y = A \times [1 - \exp(-kx)]$ , where x is the E:T ratio, y is the cytotoxic index, A is the curve maximum, and k is a constant used to calculate the slope of the best fit line. For the purposes of this study, a lytic unit was defined as the number of effector cells needed to mediate 30% lysis of target cells. The number of lytic units was calculated per 10<sup>5</sup> effector cells (LU30 per 10<sup>5</sup> cells).

#### **EXAMPLE I**

##### **Extraction of HLA-A2.1-associated Peptides from Melanoma Cells**

The human melanoma cells DM6 and DM93 were cultured in 10- chamber cell factories (Nunc, Thousand Oaks, CA) in MEM supplemented with 1% FCS and Pen-Strept. In initial experiments, the cells were harvested with 0.03% EDTA in PBS, whereas in later experiments 0.025% trypsin was also included. Trypsinization resulted in more complete harvests

and in higher cell viability without any evident change in reconstitution of epitopes or in the peptide profile (data not shown). Peptides bound to the A2 molecules were acid eluted and isolated by centrifuge filtration using a modification of the protocol described by Hunt et al. Cells were washed three times in cold PBS and solubilized in 20 ml, per  $10^9$  cells, of 1% NP-40, 0.25% sodium deoxycholate, 174 microg/ml PMSF, 5 microg/ml aprotinin, 10 microg/ml leupeptin, 16 microg/ml pepstatin A, 33 microg/ml iodoacetamide, 0.2% sodium azide, and 0.03 microg/ml EDTA. The mixture was incubated at 40C for 1 h, then centrifuged for 1 h at 100,000 x g at 40C. The supernatant was passed through a 0.22-microm filter, then was passed slowly over two protein A-Sepharose (Sigma) columns in series. The first contained GAP-A3 antibody, specific for HLA- A3, as a negative control, whereas the second column contained BB7.2, specific for HLA-A2. The columns were separately washed and eluted with 0.2N acetic acid, pH 2.7. The HLA molecules and peptides were dissociated at pH 2.1 by bringing the solution to 10% acetic acid and boiling 5 min. Peptides were separated from masses of >5 kDa (antibody, class I H chain, beta-2 microglobulin) by centrifugation through an Ultrafree-CL filter (5000 NMWL, Millipore, Bedford, MA). Yields of the peptide were estimated from the quantitation of HLA-A2.1 H chain obtained, using SDS-PAGE. The average estimated yield of HLA-A2.1 molecules was 125 microg per  $5 \times 10^9$  cells. The quantity of peptide eluted from 1 microg of HLA-A2.1 will hereafter be referred to as 1 U of peptide.

#### EXAMPLE II

##### HPLC fractionation of peptide extracts

The peptide extracts were fractionated by reversed phase high performance liquid chromatography (HPLC) on an Applied Biosystems model 130A (Foster City, CA) separation system. Peptide extracts were concentrated to 100 microl on a Speed Vac, injected onto a Brownlee narrow-bore C-18 Aquapore column (2.1 mm x 3 cm, A, 7 microm) and eluted with a 40-min gradient of 0 to 60% (v/v) acetonitrile/ 0.085% trifluor-

acetic acid (TFA) in 0.1% TFA. Fractions were collected at 1-min intervals. Cytotoxicity assays were performed to identify fractions that reconstituted CTL epitopes. For some experiments, reconstituting fractions were divided into two equal parts. The first was injected onto a Brownlee narrow-bore C-18 Aqua-pore column (2.1mm x 3 cm, 300 A, 7 microm) and eluted with a 40-min gradient of 0 to 60% (v/v) acetonitrile in 0.1% HFA that had been adjusted to pH 8.1 with 14.8 M ammonium hydroxide. The second half was injected onto the same type of column and eluted with a 40-min gradient of 0 to 60% (v/v) acetonitrile/0.1% heptafluorobutyric acid (HFBA) in 0.1% HFBA. Fractions for both second dimension separation methods were collected at 1-min intervals. Cytotoxicity assays were again performed to identify fractions that reconstitute CTL epitopes.

#### EXAMPLE III

##### Reconstitution experiments

Soluble peptide fractions were partially dehydrated on a Speed Vac, reconstituted in assay media (RPMI 1640, 10% FCS, antibiotics), then incubated for 2 h with  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled T2 cells in 150 microl/well in 96-well plates. Effector cells were added in 100 microl assay medium to give an E:T ratio of 10:1, and were incubated at 37°C. The remainder of the assay is performed as in standard chromium release assays described above. Wells containing peptide and target cells but no CTL were used as controls to rule out toxicity of the peptides themselves. Except with very acidic fractions 1 to 3, no cytotoxicity was observed with peptide alone; after the first two assays, the pH in these early fractions was adjusted to pH 7 using 1 M NaOH.

#### EXAMPLE IV

##### Generation of melanoma-specific A2.1-restricted human CTL

Lymphocytes and melanoma cells harvested from tumor-involved lymph nodes of a patient with metastatic melanoma (VMM5) were cocultured in the presence of rIL-2 and were restimulated biweekly, first with autologous and then with allogenic HLA-A2.1<sup>+</sup> melanoma. In Figure 1, the specificity

of VMM5 CTL is shown. The CTL were generated as described heretofore in Materials and Methods. Cytotoxic activity was evaluated in a 4-h chromium release assay on the HLA-A2<sup>+</sup> melanomas DM6 (solid squares) and DM93 (solid circles), the HLA-A2- melanomas DM14 (open squares) and HT144 (open circles), K562 (solid triangles) and T2 (triangles). Specific lysis of the HLA-A2.1<sup>+</sup> melanomas DM6 and DM93 was observed by day 39 of culture, whereas the HLA-A2 negative melanomas DM14 and HT144, the NK target K562, and the HLA-A2.1<sup>+</sup> lymphoblastoid Ag-processing-mutant T2 were lysed minimally. This specificity was maintained for at least 4 mo. in culture, during which time the minimal lysis of K562 and T2 diminished further. In assays against a panel of 13 targets, the specific lysis of HLA-A2.1<sup>+</sup> melanomas was confirmed, whereas HLA- A2- melanomas and A2<sup>+</sup> tumors of other cell types were not lysed. In Figure 2A, melanoma-specific CTL line VMM5 was assayed in a 4-h chromium release assay on day 94 of culture. In Figure 2B, the murine CTL clone AX21-9, specific for HLA-A2.1 molecules, was assayed. In both panels, an E:T ratio of 10:1 is represented. The name of the HLA-A2.1 transfectant of HT144 is abbreviated as HT/A2.3. Murine CTL clone AX21-9, which is specific for HLA- A2 expressed on a variety of cell types, did lyse all of the A2<sup>+</sup> targets well. These results verify that failure of VMM5 CTL to lyse the A2<sup>+</sup> nonmelanomas is not caused by inherent resistance of the targets to CTL lysis. In separate experiments, lysis of additional HLA-A2<sup>+</sup> nonmelanomas was minimal as set forth in TABLE I. As illustrated in TABLE II, the CTL lysed both fresh and cultured autologous tumor, but failed to lyse autologous PHA blasts or autologous LPS blasts.

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TABLE II

Lysis of autologous targets by VMM5 CTL and  
murine HLA-A2-specific CTL

5	% Specific	<u>Target Cell</u>	<u>Cr-51 Release</u>
<u>Effector Cell</u>			
VMM5 CTL	Fresh VMM5 melanoma	79	
	Cultured VMM5 melanoma	75	
	VMM5 PHA blasts	-1	
	VMM5 LPS blasts	-3	
10			
AT 1-15 CTL	Fresh VMM5 melanoma	62	
	Cultured VMM5 melanoma	22	
	VMM5 PHA blasts	21	
	VMM5 LPS blasts	20	

15 Lysis of the blasts by AT1-15 clones in one experiment is shown in the above table. In additional experiments, lysis of PHA blasts by AX21- 9CTL and AT-15 CTL was 80% and 25%, respectively, at an E:T ratio of 20:1, whereas lysis by the VMM5 CTL was 1%. One HLA-A2<sup>+</sup> melanoma, SkMel24, was not  
20 lysed by VMM5 CTL. The HLA-A2 negative melanoma HT144 was transfected with the A2.1 gene: the resulting transfectant, HT144 A2.03, expressed HLA-A2.1 and was susceptible to lysis by VMM5 CTL, whereas the parent line was not lysed. Thus, VMM5 CTL are a population of melanoma-specific human CTL,  
25 restricted by HLA-A2.1 molecules, that resemble other class I-MHC-restricted, human melanoma-specific CTL lines reported in the literature in that they lyse the majority of HLA-A2.1<sup>+</sup> melanomas but fail to lyse autologous nonmelanoma cells, HLA-A2<sup>+</sup> nonmelanomas, or HLA-A2<sup>-</sup> melanomas. Similar  
30 specificity for HLA-A2<sup>+</sup> melanomas has been observed with the other CTL lines studied.

## EXAMPLE V

Identification of Peptide Fractions that Reconstitute  
Melanoma-specific Epitopes

35 T2 cells were employed in the present invention to test reconstitution of melanoma-specific epitopes by soluble exogenous melanoma-derived peptides. These cells as well as

other cells expressing appropriate HLA molecules and with or without an enhanced presentation of exogenous peptides may be used as functionally equivalent in the context of the invention. In particular, these cells may be used for  
5 stimulating lymphocytes in vitro for the purpose of activating CTL for the subsequent administration to a melanoma patient.

Peptides were acid-eluted from affinity-purified HLA-A2.1 molecules isolated from detergent solubilized human  
10 melanoma cells. As graphed in Figure 3, extracts eluted from A2.1 specific (circles) or negative control immunoaffinity columns (squares) were added to  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled T2 cells. The solid symbols represent extracts plus CTL and the open symbols represent extracts alone. One unit of peptide equals  
15 that amount of peptide derived from 1 microg of HLA-A2.1 molecules. The dose of negative control extracts is based on cell equivalents, matched to the A2.1 peptide extracts. Lysis of DM93 cells (positive control) was 68%. Unfractionated peptide extract from DM93 cells did reconstitute  
20 melanoma-specific epitopes on T2 cells. Maximal lysis of 37% was achieved with 1 U of peptide. Reconstitution of CTL epitopes with unfractionated peptides, but not with negative control extracts, verified the presence in the extract of one or more peptides that reconstitute melanoma-specific  
25 epitopes.

To define further the components responsible for reconstituting CTL epitopes, the mixture of peptides extracted from the A2.1 molecules on DM6 cells was fractionated by reversed phase HPLC, and individual fractions were  
30 added to T2 cells, as shown in Figure 4. Individual HPLC fractions were added to  $2 \times 10^3$   $^{51}\text{Cr}$ -labeled T2 cells and then incubated in the presence (solid circles) or absence (open circles) of melanoma-specific CTL. Figure 4A illustrates peptides extracted from DM6 cells; Figure 4B, peptides  
35 extracted from lymphoblastoid cells JY; background lyse of T2 cells without peptide is plotted as a horizontal dotted line. Lysis of DM93 cells was 65% in A and 31% in B. Four prominent peaks of reconstitution, A, B, C, and D, located at fractions

1, 10, 14, and 16, respectively are shown in Figure 4A. A fifth, smaller, peak B1 at fraction 12 was also observed. The specificity of reconstitution with these fractions from DM6 melanoma cells was established by the inability to

5 reconstitute CTL epitopes using HPLC-fractionated peptide extracts from the A2.1 molecules expressed on the EBV-transformed lymphoblastosis cell line, JY shown in Figure 4B. This pattern of reconstitution observed with DM6-derived peptides was compared to that of another HLA-A2.1<sup>+</sup> melanoma

10 cell line, DM93, which had not been used to restimulate these CTL is illustrated in Figure 5. DM93-derived peptides of Figure 5A produced the same pattern of multiple peaks of reconstitution that was observed as with DM6 of Figure 4A. Individual HPLC fractions were added to  $2 \times 10^3$  51Cr-labeled

15 T2 cells and then incubated in the presence (closed circles) or absence (open circles) of melanoma-specific CTL. A, peptides extracted from an A2.1 specific immunoaffinity column; B, peptides extracted from a negative control immunoaffinity column. Background lysis of T2 is plotted as a

20 horizontal dotted line. Lysis of DM93 cells was 68% in A and 45% in B. From left to right peaks A, B, B1, C, and D are observed in Figure 5A. The specificity of reconstitution with the A2 extract from DM93 was confirmed by the inability to reconstitute CTL epitopes with HPLC fractions of the

25 negative control extracts, using doses comparable with those used for reconstitution with A2.1-associated peptides from the melanoma cells, as illustrated in Figure 5B.

Peptide fractions were used to evaluate reconstitution of CTL epitopes as described in Materials and Methods and il-

30 lustrated in the graph of Figure 6. The doses of peptide used were eight (solid squares), 1.25 (solid circles), and 0.25 (solid triangles) units per well. Background lysis of T2 is plotted as a horizontal dotted line. Cells were also incubated with 8 U of peptide without CTL (open squares).

35 Reconstitution for each peak was dose dependent within the range 0.25 to 8 U/well. The pattern of five peaks of reconstitution was observed in six different experiments with five different peptide extracts, using 1 to 10 U of

peptide/well. With three additional extracts, all except peak A were present. These data demonstrate that peptides present in multiple HPLC fractions common to two melanoma lines reconstitute epitopes for melanoma-specific CTL.

5 First dimension HPLC fractions that reconstituted melanoma-specific epitopes on T2 cells were fractionated a second time using either HFA (open squares) or HFBA (solid circles) as an organic modifier, and fractions were evaluated for reconstitution of CTL epitopes by addition to T2 cells,  
10 as shown in Figure 7, A to E,. In each graph, a dotted horizontal line represents the background lysis of T2 cells by CTL only. Figure 7A shows HFBA separation of 31 U of Peak A peptides from DM93 cells; Figure 7B HFA and HFBA separations of 22 U of peak B peptides from DM93; Figure 7C HFA  
15 and HFBA separations of 24 U of peak C peptides from DM93; Figure 7D HFA and HFBA separations of 22 U of peak D peptides from DM93; and Figure 7E HFA and HFBA separations of 7 U of peak D peptides from DM6. Because of its highly polar nature, peak A was separated using HFBA only. A single  
20 reconstituting peak was identified in FIGURE 7A. Second dimension separations of peaks B and D revealed a single peak of activity in both HFA and HFBA (FIGURE 7B, 7D, 7E). The activity from peak D appears at identical fractions in HFA and in HFBA for both DM6 and DM93 (FIGURE 7D, 7E). Separation  
25 of the peak C HFA resulted in two peaks of reconstitution whereas HFBA gave one broad peak (FIGURE 7C). With the resolution of peak C into two peaks, and including peak B1, a total of at least six shared CTL epitopes are demonstrated. These epitopes are labeled A2Mel-A, A2Mel-B, A2Mel-B1, A2Mel-  
30 C1, A2Mel-C2, and A2Mel-D.

In the peptide mixture responsible peak B, one peptide was identified as Sequence YMDGTMSQV, listed hereinafter as SQ. ID NO:9. The peptide is identical to a portion of the tyrosinase protein except at Position 3 where an Asp(D) is  
35 found instead of Asn(N).

## EXAMPLE VI

## Peptides Recognized by Melanoma-Specific CTL

HLA-A2.1 molecules were immunoaffinity purified from detergent lysates of the human melanoma cell line DM6. The associated peptides were released by acid extraction, separated from HLA-A2.1 and antibody by filtration, and fractionated by multiple stages of HPLC. At each stage, HPLC fractions containing relevant peptides were identified by testing their ability to reconstitute epitopes for two melanoma-specific CTL lines, VMM5 and DM204-13, after incubation with the HLA-A2.1 positive lymphoblastoid cell line T2. In Figure 8A peptides bound to A2.1 molecules were extracted and fractionated by reversed-phase HPLC, C.L. Slingluff Jr., et al, J. Immunol. (1993) 50, 2955, by using a gradient of acetonitrile/0.085% trifluoroacetic acid (TFA) in 0.1% TFA with acetonitrile increasing from 0 to 9% (0-5 min), 9 to 36% (5-55min), 36 to 60% (55-62min) (v/v), collecting one-minute fractions. In Figure 8B Fractions 2 and 3 from the separation shown in panel A were pooled and rechromatographed with a 55 minute gradient of 0 to 30% acetonitrile/0.1% heptafluorobutyric acid (HFBA) in 0.1% HFBA, collecting one-minute fractions. For both panels, peptide fractions were incubated for 2-3h with  $2 \times 10^3$   $^{51}\text{Cr}$ -labelled T2 cells in 150  $\mu\text{l}$  assay media per well in 96-well plates. CTL were added to give an effector:target ratio of 10:1, and a standard chromium release assay was conducted. The panels show lysis of target cells plus peptide by VMM5 CTL (open circles), DM204-13 CTL (closed circles) and media only (solid line without symbols). Lysis of T2 cells without peptide by VMM5 CTL was 0.3% in (A) and 0.8% in (B) and by DM204-13 CTL was 0.7% in (A) and 0.2% in (B), while positive control lysis of HLA-A2<sup>+</sup> melanoma cells by VMM5 CTL was 41% in Figure 8A and 67% in Figure 8B and by DM204-13 CTL was 28% in Figure 8A and 78% in Figure 8B.

After first dimension HPLC separation, six peaks of activity were identified for VMM5 and three for DM204-13 as shown in FIGURE 8A. Fractions 2-3 and 14-15 appeared to contain peptides recognized by both CTL lines. However,

several hundred peptides were detected by mass spectrometry in each of the active fractions in this chromatograph. Consequently, fractions 2 and 3 were pooled and submitted to a second separation using HFBA instead of TFA as the organic modifier, FIGURE 8B. Two peaks of activity were found for each CTL line, one of which contained peptides recognized by both. The most active fractions in the peak recognized by both CTL lines still contained over 50 peptides. The m/z values for a number of peptides that reconstituted epitopes for each CTL line were determined based on their presence in active fractions and absence in adjacent inactive fractions. The number of candidate peptides exceed the number that could be sequenced with available material.

Second dimension HPLC fractions 26 and 27 from Figure 8B were injected into an electrospray ionization tandem mass spectrometer using a novel online microcapillary column effluent splitter which directs the effluent simultaneously to the mass spectrometer and to the wells of a microtiter plate. Because third dimension separations using standard HPLC methods resulted in large losses of material and failed to reduce significantly the number of candidate peptides, a specific apparatus was constructed. A PRP-1 (Hamilton) microcapillary HPLC column (100  $\mu$ m by 22 cm) was butt-connected using a zero dead volume union (Valco) to two small capillaries of different lengths and interior diameters (25  $\mu$ m and 40  $\mu$ m ID, Polymicro Technologies). The column was eluted into the union with a 34 minute gradient of 0 to 60% acetonitrile. The 20  $\mu$ m capillary deposited 1/6 of the material into 50  $\mu$ l of culture media in microtiter plate wells. The larger of the two capillaries directed the remaining 5/6 of the material into the electrospray ionization source, and mass spectra of the peptides deposited in each well were recorded on a Finnigan-MAT (San Jose, CA) triple quadrupole mass spectrometer, R. A. Henderson et al., Proc. Natl. Acad. Sci. USA 90, 10275 (1993). A subsequent chromium release assay identified the wells containing peptide epitopes. Second dimension HPLC fractions 26 and 27 were pooled and analyzed using this apparatus. Both CTL

lines showed a single peak of activity. Mass spectra showed that these fractions contained approximately 50 peptides (FIGURE 9A), but the relative abundances of only three of these (with  $m/z$  values of 1046, 946 and 864) matched the activity profile for DM204-13 (FIGURE 9B). In Figure 9A, the summation of mass spectra recorded on peptides deposited in well 41 are illustrated. Many of these were eliminated as candidates because their relative abundance failed to correlate with the observed lysis. In Figure 9B the bold solid line indicates percent lysis as determined by chromium release assay. Peptide amount, as indicated by ion abundance, is plotted for  $m/z$  1046 (open circles),  $m/z$  946 (solid circles), and  $m/z$  864 (open squares)

Collision-activated dissociation analyses performed on the  $(M+H)^+$  ions for each of these three peptides defined their amino acid sequences as:  $m/z$  864, SMAPGNTSV; 946 YXEPGPVTA, where X = L or I; and 1046, AXYDATYET. The sequenced peptides were synthesized, using an equimolar mixture of Leu and Ile in place of X. The ability to reconstitute an epitope for melanoma specific CTL VMM5, and DM204-13, illustrated in Figures 10A and 10B, is shown for peptides 946 (closed squares), 864 (open squares), and 1046 (open circles). Lysis of T2 without peptide is represented by a horizontal dotted line. As shown in Figure 10, peptide 946 reconstituted the epitopes for both VMM5 and DM204-13, with half-maximal reconstitution achieved between 1 and 10 pM. When tested independently, both YLEPGPVTA and YIEPGPVTA effectively reconstituted the epitope for VMM5 at similar concentrations. Peptides 864 and 1046 had no effect at concentrations up to 10 nM. The amount of peptide 946 present in well 41 in the experiment shown in Figure 9B corresponded to a concentration of 8pM, indicating that the synthetic 946 peptide sensitized at doses comparable to that of the naturally occurring species.

Peptide 946 reconstitutes T cell recognition at concentrations that are at least two orders of magnitude lower than those of several optimized HLA-A2.1 restricted epitopes of viral or cellular origin. The antigenicity of

this peptide could be explained by high affinity for the MHC or high affinity of the TcR. The ability of the test peptides to compete with the radiolabeled standard peptide FLPSDYFPSV for binding to purified HLA-A2.1 molecules was measured using an equilibrium binding assay, J. Ruppert et al., Cell 74, 929 (1993) and Y. Chen et al., J. Immunol. in press (1994) with (Open Squares) YLEPGPVTA (peptide 946L); (solid diamonds) YIEPGPVTA (peptide 946I); (solid squares) ALWGFFPVL, another endogenous peptide isolated from HLA-A2.1, R. A. Henderson et al., Proc. Natl. Acad. Sci. USA 90, 10275 (1993); (open diamonds), APRTVALTA, an endogenous peptide isolated from HLA-B7, E. L. Huczko et al., J. Immunol. 151, 2572 (1993). The concentrations giving 50% inhibition of binding of a standard peptide to purified HLA-A2.1 molecules were 1.06 microM and 13.7 microM for the Leu and Ile version of the 946 peptide, respectively as illustrated in the graph of Figure 11. While the Leu version of 946 contains the predicted motif residues at positions 2 and 9 that support peptide binding to HLA-A2.1, the substitution of Ile at position 2 is expected to lower affinity by about a factor of 10. However, both of these values lie well outside the 5-30nM range observed for several other naturally processed peptides, and indicate the 946 isomers have considerably lower affinities. This may be due in part to the presence of a negatively charged residue at position 3, which is predicted to have a detrimental effect on binding. In keeping with the low affinity of peptide 946 for HLA-A2.1, this molecule is not present in high copy number on the cell surface. The sequence of 946 was obtained from 15 fmol of peptide present in a second dimension HPLC fraction representative of  $4 \times 10^{10}$  DM6 cells. Assuming a 5% overall yield through purification and extraction and 3 HPLC separations, it is calculated that 946-HLA-A2.1 complexes are present at only 5 copies per melanoma cell. This number is well below the 50-200 copies/cell estimated to be necessary for T cell recognition. It is conceivable that, due to the low affinity of 946 for the HLA-A2.1 molecule, the peptide may have been disproportionately lost during the washes that accompany the affinity

purification procedure. Regardless, the ability of this peptide to sensitize for CTL-mediated lysis at concentrations that are  $10^4$  -  $10^5$  lower than the  $IC_{50}$  value indicates that CTL lines VMM5 and DM204-13 have an extremely high affinity for this epitope.

To determine whether peptide 946 was a generally relevant epitope for HLA-A2.1 restricted melanoma specific CTL, lines from 3 additional patients were tested. CTL lines VMM5 and VMM6 were generated by stimulation with autologous tumor. VMM5 CTL were maintained on allogeneic A2.1+ melanoma DM6 after specificity was obtained, C. L. Slingluff Jr. et al., J. Immunol. 150, 2955 (1993). DM169-13 and DM204-13 CTL were generated by stimulation with allogeneic A2.1+ melanoma DM13, N. J. Crowley et al., J. Immunol. 146, 1692 (1991), then maintained with DM6. LD1 CTL were stimulated on a rotating schedule of stimulating allogeneic A2.1+ melanomas DM6, DM13, and DM93. DM93 is an HLA-A2.1+ melanoma used as a positive control. Peptides were synthesized by solid phase Fmoc chemistry on Wang resins with a Gilson (Middleton, WI) Model AMS 422 multiple peptide synthesizer. T2 cells were preincubated with the peptides whose (M+H)<sup>+</sup> values are indicated in the legend, Figure 12. Peptide 946 was used at a concentration of 0.1 nM, except 1 nM for DM169. All other peptides were used at a concentration of 10 nM except 250 nM for DM169 and 0.1 nM for LD1. Asterisks (\*) mark peptides not evaluated. The sequences of the peptides 864 (Seq. ID No. 88), 946 (SEQ. ID NO.:39), 1046 (SEQ. ID NO.:89) are described in the text. Peptide 1030 has the sequence YMNGTMSQV, (peptide from tyrosinase molecule). The 946 peptide, at concentrations of 0.1-1nM, shown in Figure 12, reconstituted recognition by each of these 3 lines, while neither 864 nor 1046 had any detectable activity at equal or higher concentrations. Dose response curves obtained for two of these lines showed half- maximal lysis in the 1 - 10 pM range. These results establish that 5 out of 5 patients examined had high affinity t-cells that recognized the HLA-A2.1-associated peptide 946. An additional peptide YMNGTMSQV, which originates from tyrosinase and had been

identified by Boon and colleagues as an HLA-A2.1 restricted melanoma specific T cell epitope, was also evaluated. None of 4 CTL lines recognized cells incubated with this peptide.

These five CTL lines were established in two different laboratories using varied stimulation protocols and several different stimulating tumors. Most were stimulated initially with autologous tumor (VMM6, VMM5) or allogeneic A2.1<sup>+</sup> melanoma DM13 (DM169-13, DM204-13), although many were maintained on DM6 after specificity was obtained. None of the CTL lines was stimulated exclusively with DM6, the line from which the 946 peptide was identified. The VMM6 CTL line was stimulated exclusively with autologous fresh cryo-preserved tumor. Its recognition of 946 is evidence that the T-cell response to that peptide does not require stimulation with DM6, and that fresh VMM6 melanoma cells must present the 946 peptide in a manner that induces a CTL response. The fact that all five lines recognize this 946 peptide despite their varied origins is strong evidence that this peptide may be capable of stimulating a CTL response in a large number of HLA-A2.1<sup>+</sup> individuals.

The Leu containing version of peptide 946 ( SEQ. ID NO.:14) was found in a protein identified as Pmel-17, B.S. Kwon et al., Proc. Natl. Acad. Sci. USA 88, 9228 (1991; B.S. Kwon et al., Molecular Biology and Medicine 4, 339 (1987)). Pmel-17 is a 645 amino acid protein expressed in melanocytes and melanoma and has not been detected in nonpigmented cells, B.S. Kwon et al., Proc. Natl. Acad. Sci. USA 88, 9228 (1991; B.S. Kwon et al., Molecular Biology and Medicine 4, 339 (1987)). Although its function is unknown, it has been postulated to be a component of the melanin biosynthetic pathway, B.S. Kwon et al., Proc. Natl. Acad. Sci. USA 88, 9228 (1991; B.S. Kwon et al., Molecular Biology and Medicine 4, 339 (1987)). Its presence in melanocytes as well as melanoma is consistent with the observation that some melanoma specific CTL clones recognize melanocytes, A. Anichini et al, J. Exp. Med. 177, 989 (1993). This observation, coupled with the fact that spontaneous remissions of human melanoma have been observed in conjunction with the simultaneous development of

vitiligo; T.C. Everson and W.H. Cole, Eds., Spontaneous Regression of Cancer (W. B. Saunders Company, Philadelphia, 1966), suggests that an autoimmune response directed against melanocytes may be a natural accompaniment to the development of immunity to melanoma. Although we do not yet have evidence that 946-specific CTL recognize normal melanocytes, the possibility that 946 is one of the epitopes responsible for cross-reactivity of melanoma-specific CTL with normal melanocytes raises questions about the relationship between tumor immunity and autoimmunity.

#### EXAMPLE VII

After identification of a melanoma specific CTL epitope from the protein PMEL17 (Tyr-Xaa-Glu-Pro-Gly-Pro-Val-Thr-Ala, wherein Xaa is Ile or Leu) (PEPTIDE 946I or 946L), other possible epitopes consisting of 9, 10, or 11 amino acids from PMEL17 were synthesized. To select possible epitopes, A2.1 motif information previously generated previously was used. A Gilson AMS 422 Multiple Peptide Synthesizer was used to make the synthetic peptides, which permits synthesis of only 48 peptides at one time. Due to the need to synthesize other peptides, the first set of peptides derived from PMEL17 was synthesized and contained 38 nonamers with Leu, Ile, or Met at position 2 and Leu, Ile, Val, or Ala at position 9. The sequences of these nonamers are shown below. They include the two versions of the biologically active peptide Tyr-Xaa-Glu-Pro-Gly-Pro-Val-Thr-Ala, wherein Xaa = Leu or Ile, one version with Leu at Position 2 (Seq. Id. No. 14) and one version with Ile at Position 2 (Seq. Id. No. 39). The second batch synthesized included nonamers with Thr at the ninth position, as well as 10 and 11 mers with Leu or Met at position 2 and Leu, Ile, Val, Ala, or Thr at position 9. Not

all of the 11 mers with this motif were synthesized. The list of these peptides is also shown below.

## EXAMPLE VIII

Peptides synthesized

5	9-mers	
	SEQ. ID. NO. 1	Asp-Leu-Val-Leu-Lys-Arg-Cys-Leu-Leu
	SEQ. ID. NO. 2	Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala
	SEQ. ID. NO. 3	His-Leu-Ala-Val-Ile-Gly-Ala-Leu-Leu
	SEQ. ID. NO. 4	Leu-Leu-Ala-Val-Gly-Ala-Thr-Lys-Val
10	SEQ. ID. NO. 5	Gln-Leu-Tyr-Pro-Glu-Trp-Thr-Glu-Ala
	SEQ. ID. NO. 6	Val-Ile-Trp-Val-Asn-Asn-Thr-Ile-Ile
	SEQ. ID. NO. 7	Val-Leu-Gly-Gly-Pro-Val-Ser-Gly-Leu
	SEQ. ID. NO. 8	Gly-Leu-Ser-Ile-Gly-Thr-Gly-Arg-Ala
	SEQ. ID. NO. 9	Tyr-Met-Asp-Gly-Thr-Met-Ser-Gln-Val
15	SEQ. ID. NO. 10	Ser-Ile-Gly-Thr-Gly-Arg-Ala-Met-Leu
	SEQ. ID. NO. 11	Met-Leu-Gly-Thr-His-Thr-Met-Glu-Val
	SEQ. ID. NO. 12	Gln-Leu-His-Asp-Pro-Ser-Gly-Tyr-Leu
	SEQ. ID. NO. 13	Thr-Leu-Ile-Ser-Arg-Ala-Pro-Val-Val
	SEQ. ID. NO. 14	Tyr-Leu-Glu-Pro-Gly-Pro-Val-Thr-Ala
20	SEQ. ID. NO. 15	Gly-Met-Thr-Pro-Glu-Lys-Val-Pro-Val
	SEQ. ID. NO. 16	Gly-Met-Thr-Pro-Ala-Glu-Val-Ser-Ile
	SEQ. ID. NO. 17	Ser-Ile-Thr-Gly-Ser-Leu-Gly-Pro-Leu
	SEQ. ID. NO. 18	Pro-Leu-Leu-Asp-Gly-Thr-Ala-Thr-Leu
	SEQ. ID. NO. 19	Thr-Leu-Arg-Leu-Val-Lys-Arg-Gln-Val
25	SEQ. ID. NO. 20	Arg-Leu-Val-Lys-Arg-Gln-Val-Pro-Leu
	SEQ. ID. NO. 21	Asp-Ile-Val-Gln-Gly-Ile-Glu-Ser-Ala
	SEQ. ID. NO. 22	Val-Leu-Pro-Ser-Pro-Ala-Cys-Gln-Leu
	SEQ. ID. NO. 23	Ser-Leu-Ala-Asp-Thr-Asn-Ser-Leu-Ala
	SEQ. ID. NO. 24	Ser-Leu-Ala-Val-Val-Ser-Thr-Gln-Leu
30	SEQ. ID. NO. 25	Gln-Leu-Ile-Met-Pro-Val-Pro-Gly-Ile
	SEQ. ID. NO. 26	Leu-Ile-Met-Pro-Val-Pro-Gly-Ile-Leu
	SEQ. ID. NO. 27	Ile-Met-Pro-Val-Pro-Gly-Ile-Leu-Leu
	SEQ. ID. NO. 28	Gly-Ile-Leu-Leu-Thr-Gly-Gln-Glu-Ala
	SEQ. ID. NO. 29	Leu-Leu-Thr-Gly-Gln-Glu-Ala-Gly-Leu
35	SEQ. ID. NO. 30	Gly-Leu-Gly-Gln-Val-Pro-Leu-Ile-Val
	SEQ. ID. NO. 31	Pro-Leu-Ile-Val-Gly-Ile-Leu-Leu-Val
	SEQ. ID. NO. 32	Leu-Ile-Val-Gly-Ile-Leu-Leu-Val-Leu

	SEQ. ID. NO. 33	Gly-Ile-Leu-Leu-Val-Leu-Met-Ala-Val
	SEQ. ID. NO. 34	Ile-Leu-Leu-Val-Leu-Met-Ala-Val-Val
	SEQ. ID. NO. 35	Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu
	SEQ. ID. NO. 36	Leu-Met-Ala-Val-Val-Leu-Ala-Ser-Leu
5	SEQ. ID. NO. 37	Arg-Leu-Met-Lys-Gln-Asp-Phe-Ser-Val
	SEQ. ID. NO. 38	Pro-Ile-Gly-Glu-Asn-Ser-Pro-Leu-Leu
	SEQ. ID. NO. 39	Tyr-Ile-Glu-Pro-Gly-Pro-Val-Thr-Ala
10 mers		
	SEQ. ID. NO. 40	Val-Leu-Lys-Arg-Cys-Leu-Leu-His-Leu-Ala
10	SEQ. ID. NO. 41	Cys-Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala
	SEQ. ID. NO. 42	Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala-Leu
	SEQ. ID. NO. 43	His-Leu-Ala-Val-Ile-Gly-Ala-Leu-Leu-Ala
	SEQ. ID. NO. 44	Ala-Leu-Leu-Ala-Val-Gly-Ala-Thr-Lys-Val
	SEQ. ID. NO. 45	Trp-Leu-Gly-Val-Ser-Arg-Gln-Leu-Arg-Thr
15	SEQ. ID. NO. 46	Arg-Leu-Asp-Cys-Trp-Arg-Gly-Gly-Gln-Val
	SEQ. ID. NO. 47	Ser-Leu-Lys-Val-Ser-Asn-Asp-Gly-Pro-Thr
	SEQ. ID. NO. 48	Ala-Leu-Asn-Phe-Pro-Gly-Ser-Gln-Lys-Val
	SEQ. ID. NO. 49	Ala-Met-Leu-Gly-Thr-His-Thr-Met-Glu-Val
	SEQ. ID. NO. 50	Met-Leu-Gly-Thr-His-Thr-Met-Glu-Val-Thr
20	SEQ. ID. NO. 51	Pro-Leu-Ala-His-Ser-Ser-Ser-Ala-Phe-Thr
	SEQ. ID. NO. 52	Ala-Leu-Asp-Gly-Gly-Asn-Lys-His-Phe-Leu
	SEQ. ID. NO. 53	Phe-Leu-Arg-Asn-Gln-Pro-Leu-Thr-Phe-Ala
	SEQ. ID. NO. 54	Gln-Leu-His-Asp-Pro-Ser-Gly-Tyr-Leu-Ala
	SEQ. ID. NO. 55	Tyr-Leu-Ala-Glu-Ala-Asp-Leu-Ser-Tyr-Thr
25	SEQ. ID. NO. 56	Thr-Leu-Ile-Ser-Arg-Ala-Pro-Val-Val-Thr
	SEQ. ID. NO. 57	Pro-Leu-Thr-Ser-Cys-Gly-Ser-Ser-Pro-Val
	SEQ. ID. NO. 58	Thr-Leu-Ala-Glu-Met-Ser-Thr-Pro-Glu-Ala
	SEQ. ID. NO. 59	Gly-Met-Thr-Pro-Ala-Glu-Val-Ser-Ile-Val
	SEQ. ID. NO. 60	Val-Leu-Ser-Gly-Thr-Thr-Ala-Ala-Gln-Val
30	SEQ. ID. NO. 61	Ser-Leu-Gly-Pro-Leu-Leu-Asp-Gly-Thr-Ala
	SEQ. ID. NO. 62	Leu-Leu-Asp-Gly-Thr-Ala-Thr-Leu-Arg-Leu
	SEQ. ID. NO. 63	Val-Leu-Tyr-Arg-Tyr-Gly-Ser-Phe-Ser-Val
	SEQ. ID. NO. 64	Glu-Leu-Thr-Val-Ser-Cys-Gln-Gly-Gly-Leu
	SEQ. ID. NO. 65	Gly-Leu-Pro-Lys-Glu-Ala-Cys-Met-Glu-Ile
35	SEQ. ID. NO. 66	Val-Leu-Pro-Ser-Pro-Ala-Cys-Gln-Leu-Val
	SEQ. ID. NO. 67	Ser-Leu-Ala-Asp-Thr-Asn-Ser-Leu-Ala-Val
	SEQ. ID. NO. 68	Ser-Leu-Ala-Val-Val-Ser-Thr-Gln-Leu-Ile
	SEQ. ID. NO. 69	Gln-Leu-Ile-Met-Pro-Val-Pro-Gly-Ile-Leu

- 5 SEQ. ID. NO. 70 Ile-Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu  
 SEQ. ID. NO. 71 Ile-Leu-Leu-Thr-Gly-Gln-Glu-Ala-Gly-Leu  
 SEQ. ID. NO. 72 Pro-Leu-Ile-Val-Gly-Ile-Leu-Leu-Val-Leu  
 SEQ. ID. NO. 73 Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu-Ala  
 5 SEQ. ID. NO. 74 Val-Leu-Met-Ala-Val-Val-Leu-Ala-Ser-Leu  
 SEQ. ID. NO. 75 Leu-Met-Ala-Val-Val-Leu-Ala-Ser-Leu-Ile  
 SEQ. ID. NO. 76 Gln-Leu-Pro-His-Ser-Ser-Ser-His-Trp-Leu  
 SEQ. ID. NO. 77 Val-Leu-Pro-Asp-Gly-Gln-Val-Ile-Trp-Val  
 9 mers with Thr in position 9  
 10 SEQ. ID. NO. 78 Leu-Ile-Ser-Arg-Ala-Pro-Val-Val-Thr  
 SEQ. ID. NO. 79 Val-Leu-Gln-Ala-Ala-Ile-Pro-Leu-Thr  
 SEQ. ID. NO. 80 Ser-Ile-Val-Val-Leu-Ser-Gly-Thr-Thr  
 SEQ. ID. NO. 81 Ser-Ile-Met-Ser-Thr-Glu-Ser-Ile-Thr  
 SEQ. ID. NO. 82 Ser-Leu-Gly-Pro-Leu-Leu-Asp-Gly-Thr  
 15 11 mers  
 SEQ. ID. NO. 83 Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala-  
 Leu-Leu  
 SEQ. ID. NO. 84 Cys-Leu-Leu-His-Leu-Ala-Val-Ile-Gly-  
 Ala-Leu  
 20 SEQ. ID. NO. 85 His-Leu-Ala-Val-Ile-Gly-Ala-Leu-Leu-  
 Ala-Val  
 SEQ. ID. NO. 86 Asp-Leu-Val-Leu-Lys-Arg-Cys-Leu-Leu-  
 His-Leu  
 SEQ. ID. NO. 87 Val-Leu-Lys-Arg-Cys-Leu-Leu-His-Leu-  
 25 Ala-Val  
 Additional sequences  
 SEQ. ID. NO. 88 Ser-Met-Ala-Pro-Gly-Asn-Thr-Ser-Val  
 SEQ. ID. NO. 89 Ala-Xaa-Tyr-Asp-Ala-Thr-Tyr-Glu-Thr,  
 wherein Xaa=Leu or Ile.

30 In order to identify active peptides in the complex  
 mixture obtained from the melanoma cells described above, it  
 was necessary to find a way to split a column of liquid  
 flowing at a rate of 840 nl/min into two equal parts without  
 introducing turbulence (dead volume) that would destroy the  
 35 chromatographic separation achieved in the microcapillary  
 column. Commercially available low-dead volume connections  
 were evaluated and found to be totally unsatisfactory in the  
 above regard. Fortunately, the system shown in Figure 13

worked beautifully. Chromatographic resolution was found to be the same with or without the splitter in the system. The splitter is rapid to assemble and are extremely dependable. The splitter consists of a 250 microm bore SS union.

- 5 Inserted into one side is a PEEK ferrule and nut containing a 350 microm o.d. x 100 microm i.d. fused silica column packed with POROS beads and stuffed into Teflon tubing (1/16 inch x 3 mm). Inserted into the other side is a second PEEK ferrule with SS nut containing two equal lengths of 140 uicrom o.d. x
- 10 25 microm i.d. silylated fused silica tubing also stuffed into Teflon tubing (1/16 inch x 0.3 mm). Compression of the teflon tubing creat4es the zero dead volume union. The system is illustrated in Figures 13 and 14.

- Electrospray ionization tandem mass spectrometry system
- 15 130 with online microcapillary column effluent splitter 141 to direct the effluent simultaneously to the mass spectrometer 132 and to the wells of a microtiter plate 134. A microcapillary HPLC column 140 (typically 100  $\mu$ m by 22cm) was butt connected using a zero dead volume union 144 (Valco)
- 20 to two small capillaries 148 and 149 of different lengths and interior diamters (typically 25  $\mu$ m and 40  $\mu$ m ID, Polymicro Technologies). Typically the column was eluted with an appoximate flow rate of 500 nl/min into the union with a 34 minute gradient of 0 to 60% acetonitrile. The larger of the
- 25 two capillaries directed 5/6 of the material into the electrospray ionization source (Analytica) and mass spectra were recorded on the material using a Finnigan-MAT TSQ-700 (San Jose, California) triple quadrupole mass spectrometer. The 20  $\mu$ m capillary deposited the remaining 1/6 of the
- 30 material into 50  $\mu$ l of media in microtiter plate wells. This allows deposition of a few nanoliters of eluent into a well without loss of chromatographic resolution. Timing of the splitter is adjusted so that m/z values of the peptides are recorded at the instant in which they are deposited into the
- 35 well, providing a record fo the peptides present in each well. The chromium release assay described in Figure 1 was used to determine which well contained the peptide portion of the epitope.

The above peptides were tested for activity in 51Cr release assay using the procedures set forth supra, and there was preliminary evidence that several had biologic activity as CTL epitopes. However, when confirmatory experiments were performed, the only peptides with substantial VMM6 (HLA-A2.1-restricted melanoma-specific) CTL stimulatory activity were peptides 946I (SEQ ID NO: 39) and 946L (SEQ ID NO: 14). This demonstrates the significance and serendipity of the discovery of the activity of the latter two peptides. The following table examines the CTL stimulatory activity of 946I and 946L at different concentrations:

		% specific release (of Cr-51)			
		1 $\mu$ M	1 nM	1 pM	1 fM
	T2+946-I	70.0	58.1	44.1	43.3
15	T2+946-L	69.4	73.9	53.6	52.0
	T2+946-I				
	(no CTL)	0.1	-0.9	-1.4	-0.4
	T2+946-L				
	(no CTL)	-1.4	-1.7	-1.4	-1.8

Background lysis of T2 melanoma cells by the CTL was 29%. Positive control lysis, of HLA-A2+ melanoma cell line DM6, was 72%. Thus, half-maximal lysis would be halfway between 29% and 70%-72%, or 50%; half-maximal lysis was observed between 1pM and 1nM for 946I, and at 1fM for 946L. In other assays, 1pM has given half-maximal lysis for a 946L/I mix.

#### EXAMPLE VIII

The naturally occurring peptide YMDGTMSQV was tested for recognition by the tyrosinase specific CTL clone IVSB, which had been used to identify the genetically encoded peptide YMNGTMSQV. T. Wolfei, et al., Eur. J. Immuno. 24, 759 (1994). Interestingly, YMDGTMSQV sensitized target cells for lysis at a 100-fold lower concentration than did YMNGTMSQV (half maximal lysis with 0.1 $\mu$ M and 10 $\mu$ M, respectively). Even when target cells were pretreated with the monoclonal antibody MA2.1 in order to facilitate exogenous peptide binding to HLA-A2.1. T. Wolfei, et al., Eur. J. Immunol. 24,

759 (1994; H. Bodmer, et al., Nature 342, 443 (1989), the concentration of YMDGTMSQV required to give half-maximal target cell lysis was greater than 1  $\mu$ M. This is significantly higher than that observed for numerous other peptide epitopes. R.A. Henderson, et al., Proc. Natl. Acad. Sci. USA 90, 10275 (1993); A.L. Cox, et al., Science 264, 716 (1994); J. Bertoletti, et al., J. Virol. 67, 2376 (1993); U. Utz, et al., J. Immunol. 149, 214 (1992); M.A. Bednarek, et al., ibid. 147, 4047 (1991).

To determine whether differences in target cell sensitizing activity were due to differences in the ability of the individual peptides to bind to HLA-A2.1 molecules, the binding affinity of the two peptides was measured using a quantitative binding assay. Y. Chen, et al., J. Immunol. 152, 2874 (1994); J. Ruppert, et al., Cell 74, 929 (1993). Inhibition of the binding of a standard peptide to purified HLA-A2.1 molecules was observed at similar concentrations of YMDGTMSQV and YMDGTMSQV. Thus, the asparagine and aspartic acid residues at position three of these peptides have either a similar or no influence on peptide binding to HLA-A2.1 and differences in binding affinity do not account for the difference in peptide recognition by the CTL.

Preferential CTL recognition of the naturally processed YMDGTMSQV suggests that the T cell receptor expressed by the tyrosinase specific CTL clone IVSB has a greater affinity for this species. One possible explanation of these observations is that this aspartic acid containing peptide is derived from a previously undescribed allelic variant of tyrosinase or a mutated tyrosinase gene. Although asparagine has been found at this position (residue 371 of the precursor protein) in published sequences of human tyrosinase, B.S. Kwon, et al., Proc. Natl. Acad. Sci USA 84, 7473 (1987); B. Bouchard, et al., J. Exp. Med. 169, 2029 (1989); V. Brichard, et al., J. Exp. Med. 180, 35 (1994), an allelic form containing a threonine substitution at this position has been reported in oculocutaneous albino patients, W.S. Oetting, et al., J. Invest. Dermatol. 97, 15 (1991). The naturally processed peptide YMDGTMSQV was identified in HLA-A2.1 associated

peptides extracted from two melanoma cell lines DM6 and DM93, A. Cox, et al., unpublished results, and the tyrosinase genes in these cell lines have not been sequenced. Another possibility is that this peptide originates from a gene distinct from tyrosinase. Finally, the naturally processed peptide could arise from the genetically encoded tyrosinase sequence through post-translational modification.

To distinguish among these hypotheses, mass spectrometry was used to analyze the HLA-A2.1 associated peptides extracted from the melanoma cell line NA8Mel, which does not express a tyrosinase gene, and NA8Mel+tyr, which has been transfected with a tyrosinase gene encoding asparagine at position 371. Peptides YMDGTMSQV and YMNGTMSQV have molecular masses of 1032 and 1031, respectively. Analysis of the mixture of naturally processed peptides extracted from NA8Mel by microcapillary reversed-phase HPLC failed to detect any species of mass 1031-1032 at greater than  $0.17 \text{ fmol}/3 \times 10^7$  cells. However, a single major peak, corresponding to peptide(s) in the mass window 1031-1032, was detected at the level of  $200 \text{ fmol}/3 \times 10^7$  cells among the peptides extracted from NA8Mel+tyr. This result confirms that an HLA-A2.1 associated peptide of this mass was derived from the tyrosinase gene product. When synthetic YMNGTMSQV was added to the peptide extract, two distinct peaks were detected in this mass range, indicating that the naturally processed peptide did not have this sequence. Furthermore, no signal above background was discernible at the elution position of YMNGTMSQV in normal extracts of NA8Mel+tyr, indicating that this tyrosinase gene encoded peptide is not present among HLA-A2.1 associated peptides. By contrast, the synthetic peptide YMDGTMSQV did co-elute with the naturally occurring tyrosinase peptide from NA8Mel+tyr, suggesting that these peptides were identical.

Proof for this conclusion was provided by obtaining sequence information on the naturally occurring tyrosinase peptide. Collision-activated dissociation (CAD) mass spectra were recorded on  $(M+H)^+$  ions of the corresponding peptide methyl esters. The CAD spectrum representing the peptide

YMDGTMSQV, and distinct from that of YMNGTMSQV. This establishes that the peptide encoded by the tyrosinase gene has been post-translationally modified prior to its presentation by HLA-A2.1 on the surface of these cells.

5 Creation of the naturally occurring peptide YMDGTMSQV from the genetically encoded sequence involves deamidation of asparagine to aspartic acid, a process that can occur spontaneously in acidic solution. To ensure that this conversion had not occurred during MHC purification and  
10 peptide extraction, the antigen processing mutant cell line T2 was pulsed with exogenous YMNGTMSQV, and the HLA-A2.1 associated peptides were extracted. Analysis of this extract revealed a single peak in the mass range 1031-1032, corresponding to 5.1 fmol/ $1.4 \times 10^7$  cells, which was not  
15 detected among peptides extracted from unpulsed T2 cells. Microcapillary HPLC demonstrated that this peptide co-eluted with synthetic YMNGTMSQV. No peak of greater than 0.05 fmol/ $1.4 \times 10^7$  cells was observed at the elution position of synthetic YMDGTMSQV. Thus, the conversion of asparagine to  
20 aspartic acid in the naturally processed peptide did not occur during the peptide extraction procedure.

These results establish that the naturally occurring peptide corresponding to a tyrosinase epitope is distinct from that previously identified using a genetic method. This  
25 modified peptide is recognized by tyrosinase-specific human CTL more effectively than the genetically encoded peptide, and is the only one of these two peptides to be presented by HLA-A2.1 molecules on the cell surface. This also leads to the conclusion that the naturally occurring peptide  
30 represents the epitope to which the melanoma specific CTL were originally primed. The only explanation for the presence of the naturally processed species is that it arises via a post-translational modification that results in the conversion of asparagine to aspartic acid.

35 Nonenzymatic deamidation of unmodified asparagine residues has been documented for a variety of proteins and is frequently associated with the presence of an asparagine-glycine sequence as occurs at positions 371-372 in

tyrosinase. However, although the half-lives of deamidation in these proteins and peptides depend on both their sequence and structure, they are generally estimated to be of the order of days to years. YMDGTMSQV could not be detected within peptides extracted from YMNGTMSQV-pulsed T2 cells, demonstrating that spontaneous deamidation of this peptide did not occur while it was associated with HLA-A2.1 at the cell surface. In addition, complete deamidation of tyrosinase or tyrosinase-encoded peptides intracellularly during the short time required for antigen processing and presentation would be unlikely. Since the two peptides have a similar binding affinity for HLA-A2.1, this mechanism would thus predict that both would be found among the naturally processed peptides associated with this MHC molecule, and that YMNGTMSQV would predominate. Consequently, the failure to detect any significant amount of this peptide, while the quantity of YMDGTMSQV was 1,000 times greater than background, suggests that spontaneous deamidation cannot account for the generation of this peptide epitope.

Alternatively, enzymatic deamidation of asparagine to aspartate could occur through the action of peptide:N-glycanase (PNGase). This enzyme generates an aspartate through hydrolysis of the linkage between complex-type or high-mannose glycans and asparagine during degradation of N-linked glycoproteins, T. Suzuki, et al., Biochem. Biophys. Res. Com. 194(3), 1124-1130 (1993); T. Suzuki, et al., J. Biol. Chem. 269(26), 17611-17618 (1994). A less likely possibility is glycoasparaginase which has similar activity but acts preferentially on free glycoasparagine compared to peptide bound forms, N.N. Aronson, et al., FASEB 3, 2615 (1989); V. Kaartinen, et al., J. Biol. Chem. 267, 6855 (1992); I. Mononen, et al., FASEB 7, 1247 (1993). Tyrosinase is a glycoprotein that contains six potential N-glycosylation sites, V.J. Hearing, et al., Int. J. Biochem. 19, 1141 (1987); V.J. Hearing, et al., Pigment Cell Res. 2, 75 (1989). One of these includes the asparagine residue at position 371 which has been shown in the present report to undergo post-translational modification to aspartic acid. N-glycosylation

of the asparagine residue would protect it from nonenzymatic deamidation. Furthermore, the attachment of a large carbohydrate side chain would more than likely interfere with binding to HLA-A2.1, since this residue acts as a secondary anchor for peptide binding, Y. Chen, et al., J. Immunol. 152, 2874 (1994). In either case, these factors would result in the absence of the asparagine containing form of this peptide on the cell surface. Given the strong probability that the N residue in YMNGTMSQV is glycosylated, T. Ohkura, et al., Arch. Biochem. Biophys 235(1), 63 (1984), it seems most likely that this mechanism accounts for the presentation of YMDGTMSQV in association with HLA-A2.1.

It is interesting to consider how this postulated mechanism for post-translational conversion of this residue would fit into the pathway for processing and presentation of class 1 associate peptides. The conventional pathway involves the production of peptides from proteins expressed in the cytosol and subsequent transport into the ER by the TAP proteins. It is generally assumed that peptides derived from the membrane or secreted proteins are produced after these proteins are aberrantly translated in the cytosol, rather than into the ER. However, the generation of an aspartic acid containing peptide by the action of PNGase or glycosylasparaginase would necessitate synthesis of tyrosinase on ER associated ribosomes in order for it to become N-glycosylated. The sequence and location of further processing steps is unknown. PNGase is a soluble protein whose cellular location has not yet been determined, although it has virtually no activity at lysosomal pH. On the other hand, glycoasparaginase is located exclusively in lysosomes and N-glycosylated forms of tyrosinase have been observed in this compartment. In any case, tyrosinase would need to move from the ER to the subcellular compartment containing one of these enzymes to allow generation of the deglycosylated form of the peptide. Given this scenario, it also remains uncertain whether such peptides would be dependent upon the TAP complex for transport into the ER. Interestingly, a peptide epitope derived from the HIV-1 envelope protein was

shown to be generated independently of the TAP complex, whereas generation of eptitopes from the transmembrane fusion protein of measles virus, were dependent upon TAP, S.A. Hammond, et al., Nature 364, 158-161 (1993); R.S. van Binnendijk, et al., J. Exp. Med. 176, 119-128 (1992). Further investigation into the N-glycosylation/N-deglycosylation and the catabolism of tyrosinase should reveal the processes involved in formation of this naturally occurring post-translationally modified tyrosinase epitope and their significance to class 1 antigen presentation. Such a mechanism may be generally applicable to the presentation of both glycosylated and nonglycosylated peptides from membrane bound and secreted proteins.

#### REMARKS

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and

guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein.

For immunological techniques generally, see Coligan, et al, Current Protocols in Immunology (NIH: 994); Harlow and Lane, Antibodies: A laboratory Manual (Cold Spring Harbor Lab.: 1988).

An immunogen is deemed not to occur in nature, even though its component epitopes do occur in nature, if the immunogen itself, as a single molecule, does not occur in nature. For example, a conjugate of 946L to albumin does not occur in nature even though 946L is a fragment of pMel-17 which is generated by the immune system processing of pMel-17 and complexes with MHC.

The splitter technology described herein may be used in the recovery of CTL epitopes derived from antigens of tumors other than melanomas, or antigens of parasites, viruses, etc. and these epitopes may be used in a similar manner for therapy or diagnosis. The splitter, the dimensions and proportions, and other parameters of the splitter apparatus and of its operation may be altered, provided that such alterations do not substantially interfere with the recovery and identification of CTL epitopes.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- 5 (i) APPLICANT: Slingluff, Craig L, Jr.  
Engelhard, Victor H.  
Hunt, Donald F.
- (ii) TITLE OF INVENTION: Amino Acid Sequences of HLA-  
A2.1-Associated Peptides  
Isolated from Human Cells  
and Methods of Use
- 10 (iii) NUMBER OF SEQUENCES: 90
- (iv) CORRESPONDENCE ADDRESS:
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Suite 300
- 15 (C) CITY : Charlottesville
- (D) STATE : Virginia
- (E) Country : USA
- (F) ZIP : 22902
- (v) COMPUTER READABLE FORM:
- 20 (A) MEDIUM TYPE: Diskette, 3.25 inch, 720Kb  
storage
- (B) COMPUTER : IBM compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE : ASCII
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION No:
- (B) FILING DATE :
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- 30 (A) APPLICATION No:
- (B) FILING DATE :
- (viii) ATTORNEY INFORMATION:
- (A) NAME : Parker, Sheldon H.
- (B) REG. NO: 20,738
- (C) DOCKET : GC-202
- 35 (ix) COMMUNICATION INFORMATION:
- (A) TELEPHONE: 804-977-6606
- (B) TELEFAX : 804-296-7605
- (C) TELEX : Not applicable
- 40 (2) INFORMATION FOR SEQ. ID. NO. 1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- 45 (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide

- (iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
5 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
10 (ix) FEATURE: Asp-Leu-Val-Leu-Lys-Arg-Cys-Leu-Leu  
(A) NAME/KEY: SEQ. ID. NO. 1  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 2-10.  
15 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
20 homology to the sequence ID No. 14 which  
has biologic activity.  
(D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 2:  
25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
30 (ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
35 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
40 (ix) FEATURE: Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala  
(A) NAME/KEY: SEQ. ID. NO. 2.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 9-17.  
45 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,

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as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown

(ix) FEATURE: His-Leu-Ala-Val-Ile-Gly-Ala-Leu-Leu

(A) NAME/KEY: SEQ. ID. NO. 3.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 11-19.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION: There is evidence for biologic activity as an epitope for melanoma-specific cytotoxic T lymphocytes.

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
5 (vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
10 (C) UNITS: unknown  
(ix) FEATURE: Leu-Leu-Ala-Val-Gly-Ala-Thr-Lys-Val  
(A) NAME/KEY: SEQ. ID. NO. 4.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
15 database, accession number A41234),  
residues 18-26.  
(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
20 peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14 which  
has biologic activity.  
(D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
25 INFORMATION FOR SEQ. ID. NO. 5:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
30 (D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
35 (vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
40 (C) UNITS: unknown  
(ix) FEATURE: Gln-Leu-Tyr-Pro-Glu-Trp-Thr-Glu-Ala  
(A) NAME/KEY: SEQ. ID. NO. 5.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
45 database, accession number A41234),  
residues 47-55.

5 (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequenc for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

10 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 6:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment

20 (vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:

25 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Val-Ile-Trp-Val-Asn-Asn-Thr-Ile-Ile  
(A) NAME/KEY: SEQ. ID. NO. 6.  
(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234),  
30 residues 102-110.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

35 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 7:

40 (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

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(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
5 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
10 (ix) FEATURE: Val-Leu-Gly-Gly-Pro-Val-Ser-Gly-Leu  
(A) NAME/KEY: SEQ. ID. NO. 7.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 162-170.  
15 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
20 homology to the sequence ID No. 14  
which has biologic activity.  
(D) OTHER INFORMATION: There is evidence  
for biologic activity as an epitope for  
melanoma-specific cytotoxic T  
25 lymphocytes.  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 8:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Gly-Leu-Ser-Ile-Gly-Thr-Gly-Arg-Ala  
(A) NAME/KEY: SEQ. ID. NO. 8.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 169-177.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: not applicable

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: human
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STATE: adult
- (E) HAPLOTYPE:
- (F) TISSUE TYPE: melanoma
- (G) CELL TYPE:
- (H) CELL LINE: DM93 cultured melanoma line
- (I) ORGANELLE:

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME: unknown

- (A) CHROMOSOME/SEGMENT: unknown
- (B) MAP POSITION: unknown
- (C) UNITS: unknown

(ix) FEATURE: Tyr-Met-Asp-Gly-Thr-Met-Ser-Gln-Val

(A) NAME/KEY: SEQ. ID. NO. 9 (YMDGTMSQV)

(B) LOCATION: Homology is to amino acids 369-377 of the tyrosinase protein (Entry A38444 in the PIR databank; reference: Giebel LB, et al. Genomics (1991) 9:435-445.), but there is one amino acid difference (D (aspartic acid) at position 3, instead of N

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(asparagine)); so its genetic source is not certain.

(C) IDENTIFICATION METHOD: Direct identification and sequence analysis by tandem mass spectrometry evaluation of peptides eluted from HLA-A2.1 molecules of a human melanoma cell line. It is present, and prevalent, among the peptides eluted from HLA-A2.1 molecules of the melanoma cell line, DM93.

(D) OTHER INFORMATION: This sequence has homology with a portion of the melanocyte/melanoma-specific protein, tyrosinase (YMNGTMSQV is amino acids 369-377) and this sequence number 9 has been identified for a Because it differs by one amino acid from a self-peptide and because it is not derived totally from any known protein, it may be a useful target for cytotoxic T-cells.

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown

(ix) FEATURE: Ser-Ile-Gly-Thr-Gly-Arg-Ala-Met-Leu

(A) NAME/KEY: SEQ. ID. NO. 10.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 171-179.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for

peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

- 5 (D) OTHER INFORMATION:
- (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 11:
- (i) SEQUENCE CHARACTERISTICS:
- 10 (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- 15 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:
- 20 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown
- (ix) FEATURE: Met-Leu-Gly-Thr-His-Thr-Met-Glu-Val
- 25 (A) NAME/KEY: SEQ. ID. NO. 11.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 178-186.  
(C) IDENTIFICATION METHOD: This peptide was  
30 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.
- 35 (D) OTHER INFORMATION:
- (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 12:
- (i) SEQUENCE CHARACTERISTICS:
- 40 (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- 45 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment

- (vi) ORIGINAL SOURCE: not applicable
- (vii) IMMEDIATE SOURCE: not applicable
- (viii) POSITION IN GENOME:
- 5 (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown
- (ix) FEATURE: Gln-Leu-His-Asp-Pro-Ser-Gly-Tyr-Leu
- 10 (A) NAME/KEY: SEQ. ID. NO. 12.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 243-251.
- 15 (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.
- (D) OTHER INFORMATION:
- 20 (x) PUBLICATION INFORMATION: Unpublished.
- INFORMATION FOR SEQ. ID. NO. 13:
- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- (iv) ANTI-SENSE: no
- 30 (v) FRAGMENT TYPE: internal fragment
- (vi) ORIGINAL SOURCE: not applicable
- (vii) IMMEDIATE SOURCE: not applicable
- (viii) POSITION IN GENOME:
- 35 (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown
- (ix) FEATURE: Thr-Leu-Ile-Ser-Arg-Ala-Pro-Val-Val
- 40 (A) NAME/KEY: SEQ. ID. NO. 13.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 268-276.
- 45 (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its

homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.

5 INFORMATION FOR SEQ. ID. NO. 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

10

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: not applicable

15

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STATE: adult

20

(E) HAPLOTYPE:

(F) TISSUE TYPE: melanoma

(G) CELL TYPE:

(H) CELL LINE: DM6, gift from Duke University

25

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

30

(C) UNITS: unknown

(ix) FEATURE: Tyr-Leu-Glu-Pro-Gly-Pro-Val-Thr-Ala

(A) NAME/KEY: SEQ. ID. NO. 14

(B) LOCATION: not applicable

35

(C) IDENTIFICATION METHOD: This peptide was identified directly by tandem mass spectrometric analysis of peptides eluted from HLA-A2.1 molecules purified from the human melanoma cell line, DM6, and by cytotoxicity assays using a human cytotoxic T-lymphocyte line (CTL) specific for HLA-A2.1+ melanoma.

40

(D) OTHER INFORMATION: This peptide was synthesized and was found to reconstitute an epitope for multiple

melanoma-specific CTL lines. A database search identified 100% homology with a portion of the Pmel 17 protein.

(x) PUBLICATION INFORMATION: Unpublished.

5 INFORMATION FOR SEQ. ID. NO. 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

10

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

15

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

20

(C) UNITS: unknown

(ix) FEATURE: Gly-Met-Thr-Pro-Glu-Lys-Val-Pro-Val

(A) NAME/KEY: SEQ. ID. NO. 15.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 373-381.

25

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

30

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.

35 INFORMATION FOR SEQ. ID. NO. 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

40

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

- (vi) ORIGINAL SOURCE: not applicable
- (vii) IMMEDIATE SOURCE: not applicable
- (viii) POSITION IN GENOME:
- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown
- (ix) FEATURE: Gly-Met-Thr-Pro-Ala-Glu-Val-Ser-Ile
- (A) NAME/KEY: SEQ. ID. NO. 16.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 399-407.
- (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.
- (D) OTHER INFORMATION:
- (x) PUBLICATION INFORMATION: Unpublished.
- INFORMATION FOR SEQ. ID. NO. 17:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- (iv) ANTI-SENSE: no
- (v) FRAGMENT TYPE: internal fragment
- (vi) ORIGINAL SOURCE: not applicable
- (vii) IMMEDIATE SOURCE: not applicable
- (viii) POSITION IN GENOME:
- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown
- (ix) FEATURE: Ser-Ile-Thr-Gly-Ser-Leu-Gly-Pro-Leu
- (A) NAME/KEY: SEQ. ID. NO. 17.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 449-457.
- (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its

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homology to the sequence ID No. 14  
which has biologic activity.

- (D) OTHER INFORMATION: There is evidence  
for biologic activity as an epitope for  
melanoma-specific cytotoxic T lym-  
phocytes

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Pro-Leu-Leu-Asp-Gly-Thr-Ala-Thr-Leu

- (A) NAME/KEY: SEQ. ID. NO. 18.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 456-464.  
(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
5 (viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Thr-Leu-Arg-Leu-Val-Lys-Arg-Gln-Val  
10 (A) NAME/KEY: SEQ. ID. NO. 19.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 463-471.  
15 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
20 which has biologic activity.  
(D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 20:  
(i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
30 (iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
35 (viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Arg-Leu-Val-Lys-Arg-Gln-Val-Pro-Leu  
40 (A) NAME/KEY: SEQ. ID. NO. 20.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 465-473.  
45 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pm 1-17 molecule,

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as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

5

## (D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 21:

## (i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

15

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

20

## (viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown

(ix) FEATURE: Asp-Ile-Val-Gln-Gly-Ile-Glu-Ser-Ala

25

(A) NAME/KEY: SEQ. ID. NO. 21.

(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 488-496.

(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

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35

## (D) OTHER INFORMATION: None

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 22:

## (i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 9
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

45

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

- (v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
5 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Val-Leu-Pro-Ser-Pro-Ala-Cys-Gln-Leu  
(A) NAME/KEY: SEQ. ID. NO. 22.  
10 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 544-552.  
(C) IDENTIFICATION METHOD: This peptide was  
15 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
20 (D) OTHER INFORMATION: None  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 23:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
25 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
30 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
35 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Ser-Leu-Ala-Asp-Thr-Asn-Ser-Leu-Ala  
(A) NAME/KEY: SEQ. ID. NO. 23.  
40 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 570-578.  
(C) IDENTIFICATION METHOD: This peptide was  
45 identified, from the Pm 1-17 molecule,  
as fitting the consensus s quence for  
peptides binding to human HLA-A2.1.

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Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION: None

5 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

10 (C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

15 (v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

20 (B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Ser-Leu-Ala-Val-Val-Ser-Thr-Gln-Leu

(A) NAME/KEY: SEQ. ID. NO. 24.

(B) LOCATION: Precursor protein Pmel-17 (PIR  
25 database, accession number A41234),  
residues 576-584.

(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
30 peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION: None

35 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

40 (C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

45 (v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

5 (C) UNITS: unknown

(ix) FEATURE: Gln-Leu-Ile-Met-Pro-Val-Pro-Gly-Ile

(A) NAME/KEY: SEQ. ID. NO. 25.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234),

10 residues 583-591.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for

15 peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION: There is evidence for biologic activity as an epitope for

20 melanoma-specific cytotoxic T lymphocytes.

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 26:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

30 (iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

35 (viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Leu-Ile-Met-Pro-Val-Pro-Gly-Ile-Leu

40 (A) NAME/KEY: SEQ. ID. NO. 26.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234),

residues 584-592.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for

45

peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

- 5 (D) OTHER INFORMATION: None
- (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 27:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9
- 10 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- 15 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:
- 20 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown
- (ix) FEATURE: Ile-Met-Pro-Val-Pro-Gly-Ile-Leu-Leu
- (A) NAME/KEY: SEQ. ID. NO. 27.
- 25 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 585-593.
- (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
30 as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.
- 35 (D) OTHER INFORMATION: None
- (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 28:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9
- 40 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear
- (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- 45 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
5 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Gly-Ile-Leu-Leu-Thr-Gly-Gln-Glu-Ala  
(A) NAME/KEY: SEQ. ID. NO. 28.  
10 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 590-598.  
(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
15 as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
(D) OTHER INFORMATION: None  
20 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 29:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
(B) TYPE: amino acids  
25 (C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
30 (v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
35 (B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Leu-Leu-Thr-Gly-Gln-Glu-Ala-Gly-Leu  
(A) NAME/KEY: SEQ. ID. NO. 29.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
40 database, accession number A41234),  
residues 592-600.  
(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
45 as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its

homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION: None

(x) PUBLICATION INFORMATION: Unpublished.

5 INFORMATION FOR SEQ. ID. NO. 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

10 (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

15 (vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

20 (C) UNITS: unknown

(ix) FEATURE: Gly-Leu-Gly-Gln-Val-Pro-Leu-Ile-Val

(A) NAME/KEY: SEQ. ID. NO. 30.

(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 599-607.

25 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule, as  
fitting the consensus sequence for peptides  
binding to human HLA-A2.1. Pmel-17 was  
30 identified because of its homology to the  
sequence ID No. 14 which has biologic  
activity.

(D) OTHER INFORMATION: None

(x) PUBLICATION INFORMATION: Unpublished.

35 INFORMATION FOR SEQ. ID. NO. 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

40 (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

45 (vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

5 (ix) FEATURE: Pro-Leu-Ile-Val-Gly-Ile-Leu-Leu-Val  
(A) NAME/KEY: SEQ. ID. NO. 31.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 604-612.  
10 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule, as  
fitting the consensus sequence for peptides  
binding to human HLA-A2.1. Pmel-17 was  
15 identified because of its homology to the  
sequence ID No. 14 which has biologic  
activity.  
(D) OTHER INFORMATION: None  
(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 32:  
20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

25 (ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
30 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

35 (ix) FEATURE: Leu-Ile-Val-Gly-Ile-Leu-Leu-Val-Leu  
(A) NAME/KEY: SEQ. ID. NO. 32.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 605-613.  
40 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
45 homology to the sequence ID No. 14  
which has biologic activity.

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(D) OTHER INFORMATION: None

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 33:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

10 (iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

15 (viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Gly-Ile-Leu-Leu-Val-Leu-Met-Ala-Val

20 (A) NAME/KEY: SEQ. ID. NO. 33.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 608-616.

(C) IDENTIFICATION METHOD: This peptide was

25 identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its

30 homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION: There is evidence for biologic activity as an epitope for melanoma-specific cytotoxic T lymphocytes.

35 (x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

40 (C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

45 (v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
    (A) CHROMOSOME/SEGMENT: 12  
        (B) MAP POSITION: unknown  
        (C) UNITS: unknown  
5 (ix) FEATURE: Ile-Leu-Leu-Val-Leu-Met-Ala-Val-Val  
    (A) NAME/KEY: SEQ. ID. NO. 34.  
        (B) LOCATION: Precursor protein Pmel-17 (PIR  
            database, accession number A41234),  
10 residues 609-617.  
        (C) IDENTIFICATION METHOD: This peptide was  
            identified, from the Pmel-17 molecule,  
            as fitting the consensus sequence for  
            peptides binding to human HLA-A2.1.  
15 Pmel-17 was identified because of its  
            homology to the sequence ID No. 14  
            which has biologic activity.  
        (D) OTHER INFORMATION: There is evidence  
20 for biologic activity as an epitope for  
            melanoma-specific cytotoxic T lymphocytes.  
    (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 35:  
    (i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 9  
        (B) TYPE: amino acids  
        (C) STRANDEDNESS: n/a  
        (D) TOPOLOGY: linear  
    (ii) MOLECULAR TYPE: peptide  
30 (iii) HYPOTHETICAL: no  
    (iv) ANTI-SENSE: no  
    (v) FRAGMENT TYPE: internal fragment  
    (vi) ORIGINAL SOURCE: not applicable  
    (vii) IMMEDIATE SOURCE: not applicable  
35 (viii) POSITION IN GENOME:  
        (A) CHROMOSOME/SEGMENT: 12  
        (B) MAP POSITION: unknown  
        (C) UNITS: unknown  
    (ix) FEATURE: Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu  
40 (A) NAME/KEY: SEQ. ID. NO. 35.  
        (B) LOCATION: Precursor protein Pmel-17 (PIR  
            database, accession number A41234),  
            residues 610-618.  
        (C) IDENTIFICATION METHOD: This peptide was  
45 identified, from the Pmel-17 molecule,

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 232-241.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Gln-Leu-His-Asp-Pro-Ser-Gly-Tyr-Leu-Ala

(A) NAME/KEY: SEQ. ID. NO. 54.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 243-252.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Tyr-Leu-Ala-Glu-Ala-Asp-Leu-Ser-Tyr-Thr  
(A) NAME/KEY: SEQ. ID. NO. 55.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 250-259.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
25 (D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 56:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Thr-Leu-Ile-Ser-Arg-Ala-Pro-Val-Val-Thr  
(A) NAME/KEY: SEQ. ID. NO. 56.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 268-277.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Pro-Leu-Thr-Ser-Cys-Gly-Ser-Ser-Pro-Val

(A) NAME/KEY: SEQ. ID. NO. 57.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 297-306.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Thr-Leu-Ala-Glu-Met-Ser-Thr-Pro-Glu-Ala  
(A) NAME/KEY: SEQ. ID. NO. 58.  
(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 388-397.  
(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.  
(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 59:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Gly-Met-Thr-Pro-Ala-Glu-Val-Ser-Ile-Val  
(A) NAME/KEY: SEQ. ID. NO. 59.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 399-408.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Val-Leu-Ser-Gly-Thr-Thr-Ala-Ala-Gln-Val

(A) NAME/KEY: SEQ. ID. NO. 60.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 409-418.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Ser-Leu-Gly-Pro-Leu-Leu-Asp-Gly-Thr-Ala  
(A) NAME/KEY: SEQ. ID. NO. 61.  
15 (B) LOCATION: Precursor protein Pmel-17  
(PIRT database, accession number  
A41234), residues 453-462.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
25 (D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 62:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Leu-Leu-Asp-Gly-Thr-Ala-Thr-Leu-Arg-Leu  
(A) NAME/KEY: SEQ. ID. NO. 62.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 457-466.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Val-Leu-Tyr-Arg-Tyr-Gly-Ser-Phe-Ser-Val

(A) NAME/KEY: SEQ. ID. NO. 63.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 476-485.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 64:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Glu-Leu-Thr-Val-Ser-Cys-Gln-Gly-Gly-Leu  
(A) NAME/KEY: SEQ. ID. NO. 64.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 511-520.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
25 (D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 65:  
(i) SEQUENCE CHARACTERISTICS:  
30 (A) LENGTH: 10  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Gly-Leu-Pro-Lys-Glu-Ala-Cys-Met-Glu-Ile  
(A) NAME/KEY: SEQ. ID. NO. 65.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 519-528.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Val-Leu-Pro-Ser-Pro-Ala-Cys-Gln-Leu-Val

(A) NAME/KEY: SEQ. ID. NO. 66.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 544-553.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptid  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Ser-Leu-Ala-Asp-Thr-Asn-Ser-Leu-Ala-Val  
(A) NAME/KEY: SEQ. ID. NO. 67.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 570-579.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:  
25 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 68:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Ser-Leu-Ala-Val-Val-Ser-Thr-Gln-Leu-Ile  
(A) NAME/KEY: SEQ. ID. NO. 68.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 576-585.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Gln-Leu-Ile-Met-Pro-Val-Pro-Gly-Ile-Leu

(A) NAME/KEY: SEQ. ID. NO. 69.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 583-592.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 70:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Ile-Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu  
(A) NAME/KEY: SEQ. ID. NO. 70.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 609-618.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
25 (D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 71:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Ile-Leu-Leu-Thr-Gly-Gln-Glu-Ala-Gly-Leu  
(A) NAME/KEY: SEQ. ID. NO. 71.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 591-600.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Pro-Leu-Ile-Val-Gly-Ile-Leu-Leu-Val-Leu

(A) NAME/KEY: SEQ. ID. NO. 72.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 604-613.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no

5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:

10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Leu-Leu-Val-Leu-Met-Ala-Val-Val-Leu-Ala  
(A) NAME/KEY: SEQ. ID. NO. 73.

15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 610-619.  
(C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
20 peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

25 (D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 74:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 10  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no

35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:

40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Val-Leu-Met-Ala-Val-Val-Leu-Ala-Ser-Leu  
(A) NAME/KEY: SEQ. ID. NO. 74.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 612-621.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 75:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Leu-Met-Ala-Val-Val-Leu-Ala-Ser-Leu-Ile

(A) NAME/KEY: SEQ. ID. NO. 75.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 613-622.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 76:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Gln-Leu-Pro-His-Ser-Ser-Ser-His-Trp-Leu  
(A) NAME/KEY: SEQ. ID. NO. 76.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 636-645.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:  
25 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 77:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Val-Leu-Pro-Asp-Gly-Gln-Val-Ile-Trp-Val  
(A) NAME/KEY: SEQ. ID. NO. 77.

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(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 96-105.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Leu-Ile-Ser-Arg-Ala-Pro-Val-Val-Thr

(A) NAME/KEY: SEQ. ID. NO. 78.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 269-277.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

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(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Val-Leu-Gln-Ala-Ile-Pro-Leu-Thr  
(A) NAME/KEY: SEQ. ID. NO. 79.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234), residues  
291-299.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.  
25 (D) OTHER INFORMATION:  
(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 80:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear  
(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown  
(ix) FEATURE: Ser-Ile-Val-Val-Leu-Ser-Gly-Thr-Thr  
(A) NAME/KEY: SEQ. ID. NO. 80.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 406-414.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Ser-Ile-Met-Ser-Thr-Glu-Ser-Ile-Thr

(A) NAME/KEY: SEQ. ID. NO. 81.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 443-451.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 82:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
5 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
10 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Ser-Leu-Gly-Pro-Leu-Leu-Asp-Gly-Thr  
(A) NAME/KEY: SEQ. ID. NO. 82.  
15 (B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 453-461.  
(C) IDENTIFICATION METHOD: This peptide was  
20 identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:  
25 (x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 83:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 11  
30 (B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
35 (iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
40 (A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala-Leu  
-Leu  
45 (A) NAME/KEY: SEQ. ID. NO. 83.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 9-19.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 84:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 12

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE: Gys-Leu-Leu-His-Leu-Ala-Val-Ile-Gly-Ala  
-Leu

(A) NAME/KEY: SEQ. ID. NO. 84.

(B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 8-18.

(C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its homology to the sequence ID No. 14 which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 85:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

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(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

5 (iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable  
(vii) IMMEDIATE SOURCE: not applicable

10 (viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

(ix) FEATURE: His-Leu-Ala-Val-Ile-Gly-Ala-Leu-Leu-Ala  
15 -Val  
(A) NAME/KEY: SEQ. ID. NO. 85.  
(B) LOCATION: Precursor protein Pmel-17 (PIR  
database, accession number A41234),  
residues 11-21.

20 (C) IDENTIFICATION METHOD: This peptide was  
identified, from the Pmel-17 molecule,  
as fitting the consensus sequence for  
peptides binding to human HLA-A2.1.  
Pmel-17 was identified because of its  
25 homology to the sequence ID No. 14  
which has biologic activity.

(D) OTHER INFORMATION:

(x) PUBLICATION INFORMATION: Unpublished.

INFORMATION FOR SEQ. ID. NO. 86:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 11  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

35 (ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: internal fragment  
(vi) ORIGINAL SOURCE: not applicable

40 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME:  
(A) CHROMOSOME/SEGMENT: 12  
(B) MAP POSITION: unknown  
(C) UNITS: unknown

45 (ix) FEATURE: Asp-Leu-Val-Leu-Lys-Arg-Cys-Leu-Leu-His  
-Leu

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- (A) NAME/KEY: SEQ. ID. NO. 86.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 2-12.
- 5 (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its
- 10 homology to the sequence ID No. 14 which has biologic activity.
- (D) OTHER INFORMATION:
- (x) PUBLICATION INFORMATION: Unpublished.
- 15 INFORMATION FOR SEQ. ID. NO. 87:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 11
- (B) TYPE: amino acids
- (C) STRANDEDNESS: n/a
- (D) TOPOLOGY: linear
- 20 (ii) MOLECULAR TYPE: peptide
- (iii) HYPOTHETICAL: no
- (iv) ANTI-SENSE: no
- (v) FRAGMENT TYPE: internal fragment
- (vi) ORIGINAL SOURCE: not applicable
- 25 (vii) IMMEDIATE SOURCE: not applicable
- (viii) POSITION IN GENOME:
- (A) CHROMOSOME/SEGMENT: 12
- (B) MAP POSITION: unknown
- (C) UNITS: unknown
- 30 (ix) FEATURE: Val-Leu-Lys-Arg-Cys-Leu-Leu-His-Leu-Ala-Val
- (A) NAME/KEY: SEQ. ID. NO. 87.
- (B) LOCATION: Precursor protein Pmel-17 (PIR database, accession number A41234), residues 4-14.
- 35 (C) IDENTIFICATION METHOD: This peptide was identified, from the Pmel-17 molecule, as fitting the consensus sequence for peptides binding to human HLA-A2.1. Pmel-17 was identified because of its
- 40 homology to the sequence ID No. 14 which has biologic activity.
- (D) OTHER INFORMATION:
- (x) PUBLICATION INFORMATION: Unpublished.
- 45 INFORMATION FOR SEQ. ID. NO. 88:
- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9  
(B) TYPE: amino acids  
(C) STRANDEDNESS: n/a  
(D) TOPOLOGY: linear

5 (ii) MOLECULAR TYPE: peptide  
(iii) HYPOTHETICAL: no  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: not applicable  
(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: human  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STATE: adult  
(E) HAPLOTYPE:  
15 (F) TISSUE TYPE: melanoma  
(G) CELL TYPE:  
(H) CELL LINE: DM6, gift from Duke University  
(I) ORGANELLE:

20 (vii) IMMEDIATE SOURCE: not applicable  
(viii) POSITION IN GENOME: unknown  
(ix) FEATURE: Ser-Met-Ala-Pro-Gly-Asn-Thr-Ser-Val

(A) NAME/KEY: SEQ. ID. NO. 88 (Peptide 864)  
(B) LOCATION: not applicable  
25 (C) IDENTIFICATION METHOD: This peptide was  
identified directly by tandem mass  
spectrometric analysis of peptides  
eluted from HLA-A2.1 molecules purified  
from the human melanoma cell line, DM6.

30 (D) OTHER INFORMATION: This peptide was synthesized, but did not reconstitute an epitope for these CTL. A database search identified no homologous matches to known proteins.

(x) PUBLICATION INFORMATION:

35 (A) AUTHORS: Cox, AL  
Skipper, J  
Chen, Y  
Henderson, R  
Darrow, TL  
40 Shabanowitz, J  
Engelhard, VH  
Hunt, DF  
Slingsluff, CL.

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(B) TITLE: Identification of a Peptide  
Recognized by Five Melanoma-specific

Human Cytotoxic T-Cell Lines

(C) JOURNAL: Science

5 (D) VOLUME: Submitted ( A copy of the  
manuscript is enclosed with this patent  
application.)

(E) ISSUE:

(F) PAGES:

10 (G) DATE:

INFORMATION FOR SEQ. ID. NO. 89:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

15 (B) TYPE: amino acids

(C) STRANDEDNESS: n/a

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

20 (v) FRAGMENT TYPE: not applicable

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(B) STRAIN:

25 (C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STATE: adult

(E) HAPLOTYPE:

(F) TISSUE TYPE: melanoma

(G) CELL TYPE:

30 (H) CELL LINE: DM6, gift from Duke Univerr-  
sity

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE: not applicable

(viii) POSITION IN GENOME: {enter info here & ABC}

(A) CHROMOSOME/SEGMENT:

35 (B) MAP POSITION:

(C) UNITS:

(ix) FEATURE: Ala-Xaa-Tyr-Asp-Ala-Thr-Tyr-Glu-Thr,  
wherein Xaa = Leu or Ile.

(A) NAME/KEY: SEQ. ID. NO. 89 (peptide 1046)

40 (B) LOCATION: Cofilin residues 83-91. {Enter  
Data here}

(C) IDENTIFICATION METHOD: This peptide was  
identified directly by tandem mass

sp ctrometric analysis of peptides  
eluted from HLA-A2.1 molecules purified  
from the human melanoma cell line, DM6.

(D) OTHER INFORMATION: This peptide was syn-  
thesized, but did not reconstitute an

epitope for these CTL. A database  
search identified homology with  
cofilin, an actin-modulating protein  
that is ubiquitously expressed in mam-  
malian cells. The sequence, when Xaa  
is Leu, corresponds to residues 83-91  
of the cofilin protein (PIR S12632;  
ref: Ogawa, K. et al. Nucleic Acids  
Res. 18: 7169 (1990).

(x) PUBLICATION INFORMATION: Unpublished.  
INFORMATION FOR SEQ. ID. NO. 90:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: nucleic acids

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: cDNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STATE: adult

(E) HAPLOTYPE:

(F) TISSUE TYPE: melanoma

(G) CELL TYPE:

(H) CELL LINE: DM6, a gift from Duke Univer-  
sity

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE: not applicable

(A) LIBRARY:

(B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: chromosome 12

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE: TAC CTG GAG CCT GGC CCA GTC ACT GCC

(A) NAME/KEY: SEQ. ID. NO. 90 (946 gene)

(B) LOCATION: See identification method.

(C) IDENTIFICATION METHOD: The protein data bank was searched for homology to the peptide directly identified as SEQ. ID. NO. 14. Homology to the Pmel-17 protein was identified at residues 849-875. The genetic sequence encoding those residues was identified from the gene bank and is listed above.

(D) OTHER INFORMATION: The peptide encoded by this gene sequence is biologically very active as an epitope for melanoma-specific cytotoxic T lymphocytes.

(x) PUBLICATION INFORMATION: None.

INFORMATION FOR SEQ. ID. NO. 91:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2131

(B) TYPE: nucleic acids

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: cDNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: complete gene

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STATE:

(E) HAPLOTYPE:

(F) TISSUE TYPE: melanocyte

(G) CELL TYPE:

(H) CELL LINE:

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:

(A) LIBRARY:

(B) CLONE:

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: chromosome 12

(B) MAP POSITION:

(C) UNITS:

(ix) FEATURE:

(A) NAME/KEY: SEQ. ID. NO. 91

(B) LOCATION: gene for Pmel-17 (accession number M77348)

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(C) IDENTIFICATION METHOD: This gene encodes the protein Pmel-17, which was found to contain SEQ. ID. NO. 14, which has biologic activity as an epitope for melanoma-specific cytotoxic T lymphocytes.

(D) OTHER INFORMATION: This sequence has previously been described. We are claiming its use in a tumor vaccine for the prevention and treatment of melanoma.

(x) PUBLICATION INFORMATION:

(A) AUTHORS:

Kwon, B. S. et al.

(B) TITLE: A Melanocyte Specific Gene, Pmel 17, Mapped Near the Silver Coat Color Locus on Mouse Chromosome 10 and is in A Syntenic Region on Human Chromosome 12

(C) JOURNAL: Proc. Natl. Acad. Sci. USA

(D) VOLUME: 88

(E) ISSUE:

(F) PAGES: 9228:9232

(G) DATE: 1991

INFORMATION FOR SEQ. ID. NO. 92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: nucleic acids

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: cDNA

(iii) HYPOTHETICAL: yes

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE: not applicable

(A) ORGANISM:

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STATE:

(E) HAPLOTYPE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(H) CELL LINE:

(I) ORGANELLE:

(vii) IMMEDIATE SOURCE: not applicable

(A) LIBRARY:

128

(B) CLONE:

(viii) POSITION IN GENOME: not applicable

(A) CHROMOSOME/SEGMENT:

(B) MAP POSITION:

5 (C) UNITS:

(ix) FEATURE: {YMDGTMSQV}: TAT ATG GAT GGA ACA ATG TCC  
CAG GTA

(A) NAME/KEY: SEQ. ID. NO. 92 (YMDGTMSQV  
gene)

10 (B) LOCATION: not applicable

(C) IDENTIFICATION METHOD: The peptide  
YMDGTMSQV was directly identified on  
HLA-A2.1 molecules of a human melanoma  
(SEQ. ID. NO. 9). We derived a gene  
15 sequence encoding YMDGTMSQV that shares  
all the nucleic acid residues 2365 -  
2391 of the tyrosinase gene, which en-  
code YMDGTMSQV, except for a change of  
A to G at residue 2371, encoding D  
20 (Asp) at position 3 of the peptide, in-  
stead of N (Asn).

(D) OTHER INFORMATION: The reference for the  
tyrosinase sequence is Genomics 9, 435  
-445, 1991 Giebel, LB et al. Accession  
25 number for the tyrosine sequence is  
A38444 in the PIR databank.

(x) PUBLICATION INFORMATION: not published.

What is claimed is:

1. A melanoma-specific immunogen which comprises one or more melanoma-specific CTL epitopes, which may be the same or different, wherein at least one of said epitopes is at least substantially homologous with a CTL epitope of the melanoma antigen pMel-17, said immunogen itself does not occur in nature, and where in an in vitro cytotoxicity assay the concentration of at least one of said epitopes in oligopeptide form needed to stimulate CTLs so as to achieve half the maximal lysis of melanoma cells by CTLs in excess of background lysis is no more than about 1nM, more preferably no more than 100pM, still more preferably no more than 10pM, and where said immunogen elicits a CTL response which is more melanoma specific or more potent than that elicited by pMel-17 per se.
2. The immunogen of claim 1 or the peptide of claim 21 which comprises an amino acid sequence at least substantially homologous with the peptide 946L (SEQ. ID NO.:14).
3. The immunogen of claim 2 or peptide of claim 21 comprises an amino acid sequence identical to peptide 946L (SEQ. ID. NO.:14).
4. The immunogen of claim 2 or peptide of claim 21 which comprises an amino acid sequence which differs from peptide 946L (SEQ. ID NO.:14) by one or more of the following substitution mutations:
  - Tyr(1) to Phe or Trp, and/or
  - Leu(2) to Ile, Val or Met, and/or
  - Pro(4) to Ala, Ser, Thr, or Gly, and/or
  - Gly(5) to Pro, Ala, Ser, or Thr, and/or
  - Pro(6) to Ala, Ser, Thr, or Gly, and/or
  - Val(7) to Ile, Leu or Met, and/or
  - Thr(8) to Ala, Ser, Pro, or Gly, and/or
  - Ala(9) to Thr, Ser, Pro, or Gly.
5. The immunogen of claim 2 or peptide of claim 21 which comprises an amino acid sequence which differs from peptide 946L (SEQ. ID NO.:14) by a single substitution mutation.
6. The immunogen of claim 2 or peptide of claim 21 which comprises an amino acid sequence identical to peptide 946L (SEQ. ID NO.:14) or peptide 946I (SEQ. ID NO.:39).

AMENDED SHEET

7. A melanoma-specific immunogen which comprises one or more melanoma-specific epitopes, which may be the same or different, wherein at least one of the epitopes comprises an amino acid sequence which is at least substantially homologous with but different from an amino acid sequence of a segment of a tyrosinase as genetically encoded by melanoma cells, and where said immunogen elicits a CTL response which is more melanoma specific or more potent than that elicited by tyrosinase per se.

8. The immunogen of claim 7 or peptide of claim 22 which comprises an amino acid sequence which is at least substantially homologous with peptide 1030, SEQ. ID NO.:9.

9. The immunogen of claim 7 wherein at least one of said epitopes comprises the amino acid sequence of peptide 1030 SEQ. ID NO.:9.

10. The melanoma-specific immunogen of any of claims 1-9 wherein at least one of said epitopes is an HLA-A2-restricted epitope which is recognized by CTLs of at least two different HLA-A2<sup>+</sup> individuals, more preferably by CTLs of at least five different HLA-A2<sup>+</sup> individuals.

11. The immunogen of any of claims 1-10 which comprises a plurality of melanoma-specific CTL epitopes, which may be the same or different.

12. The immunogen of any of claims 1-11 which is a conjugate of said one or more epitopes to a soluble immunogenic carrier, a virus particle, or a branched lysine core structure.

13. The immunogen of any of claims 1-9, 11 or 12 in which the human CTL response to said immunogen is restricted by a class I MHC molecule serologically defined as HLA-A1, HLA-A2, HLA-A3, HLA-A11, HLA-A24, HLA-B7 or HLA-B8.

14. The immunogen of claim 2 which does not include all pMel-17 CTL epitopes.

15. The immunogen of claim 8 which does not include all tyrosinase CTL epitopes.

16. The immunogen of claim 2 wherein the only epitopes at least substantially homologous with a pMel-17 CTL epitope are those substantially homologous with peptide 946L.

17. The immunogen of claim 8 wherein the only epitopes at least substantially homologous with a tyrosinase CTL epitope

are those substantially homologous with peptide 1030.

18. The immunogen of claim 1 which comprises more copies of amino acid sequences substantially homologous to a particular melanoma specific CTL-epitope of pMel-17 than are found natively  
5 in pMel-17.

19. The immunogen of claim 18 which comprises two or more amino acid sequences substantially homologous with peptide 946L.

20. An expression vector which comprises a gene encoding  
10 a melanoma-specific immunogen according to any of claims 1-19, or a peptide according to claims 21-22, operably linked to one or more expression control sequences, whereby said gene may be expressed in a mammalian subject, in which subject said expressed immunogen or peptide is capable of eliciting a  
15 melanoma-specific CTL response.

21. An isolated, purified, or synthetic peptide of about 9 to about 15 residues in length, more preferably 9-11 residues in length, which is at least substantially homologous with a melanoma-specific pMel-17 CTL epitope.

20 22. An isolated, purified or synthetic peptide of about 9 to about 15 residues in length, more preferably 9-11 residues in length, which is at least substantially homologous with a melanoma-specific tyrosinase epitope.

23. A composition comprising a peptide according to any  
25 of claims 21-22 and a class I MHC molecule, whereby T lymphocytes may be stimulated by said peptide.

24. T lymphocytes stimulated by a peptide according to claims 21 or 22 or an immunogen according to any of claims 1-19.

25. Use of the immunogen of any of claims 1-19, the  
30 peptides of any of claims 21-22, the vector of claim 20, or the stimulated T lymphocytes of claim 24 in the manufacture of a composition for at least partial protection of mammals, such as humans, against melanoma.

26. An apparatus for use in the simultaneous analysis  
35 of a single effluent of a microcapillary column, in a mass spectrometer and in a microtiter plate comprising a microcapillary high pressure liquid chromatography column, a zero dead volume union and two capillaries, said micro-capillary high

pressure liquid chromatography column being an inlet to said zero dead volume union and said two capillaries being connected to said union to provide split flow of said effluent into two streams, one stream being to a mass spectrometer and the other  
5 stream being to a microtiter plate.

27. Use of the apparatus of claim 26 in the identification of CTL epitopes, where peptides are eluted from an MHC complex and separated on said microcapillary column.

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AMENDED SHEET

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FIG. 1

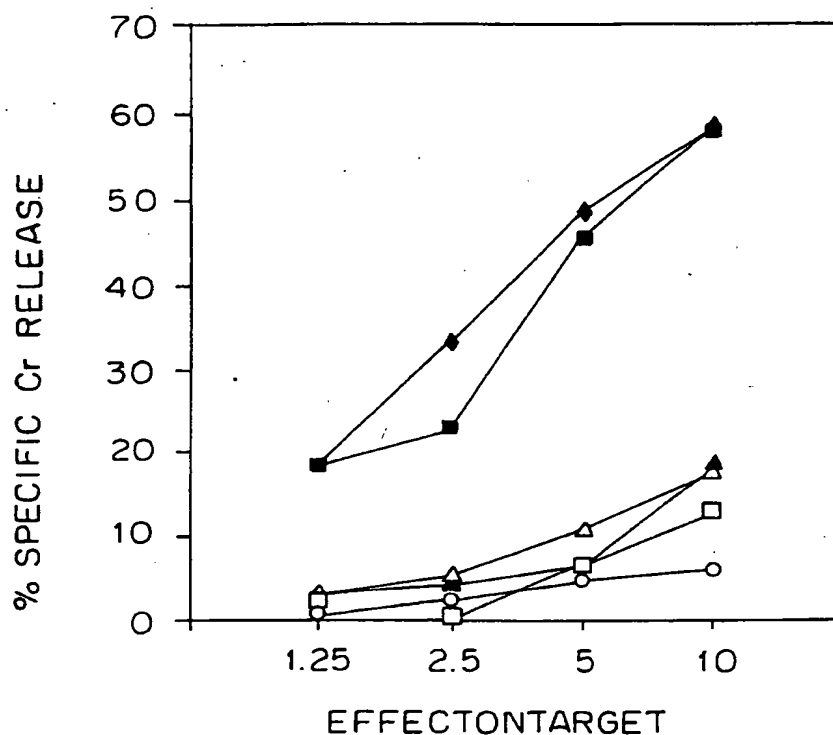


FIG. 2A

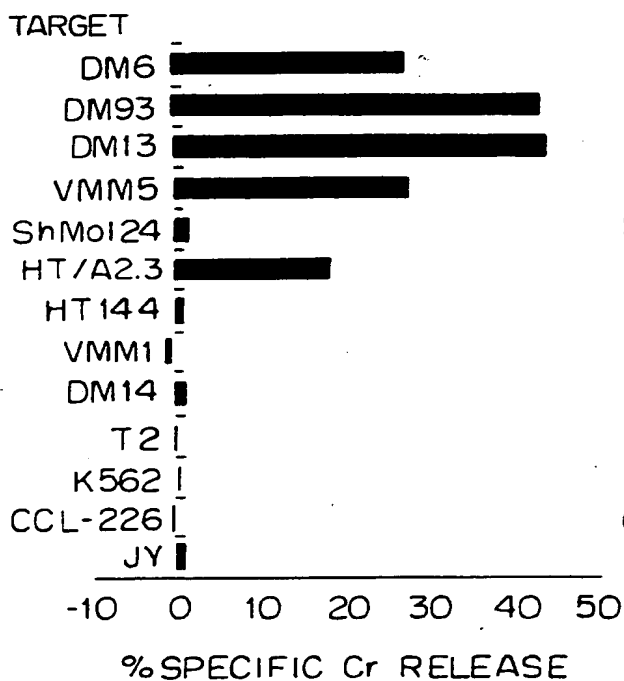
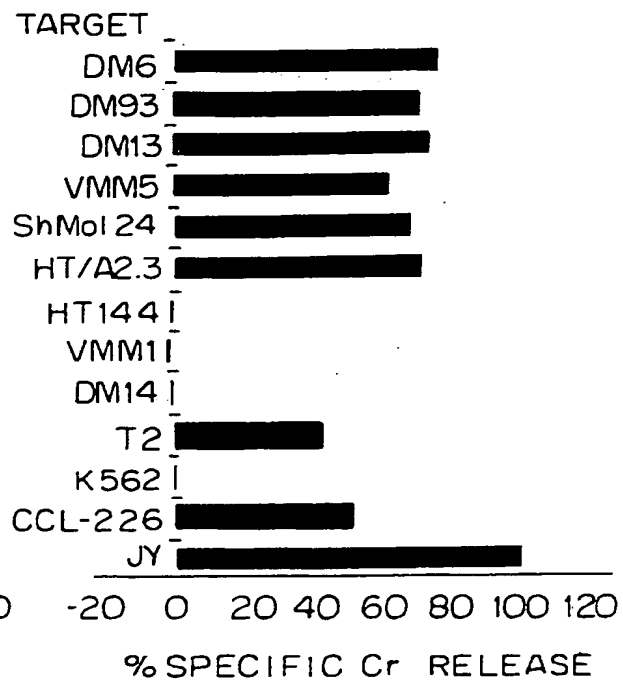
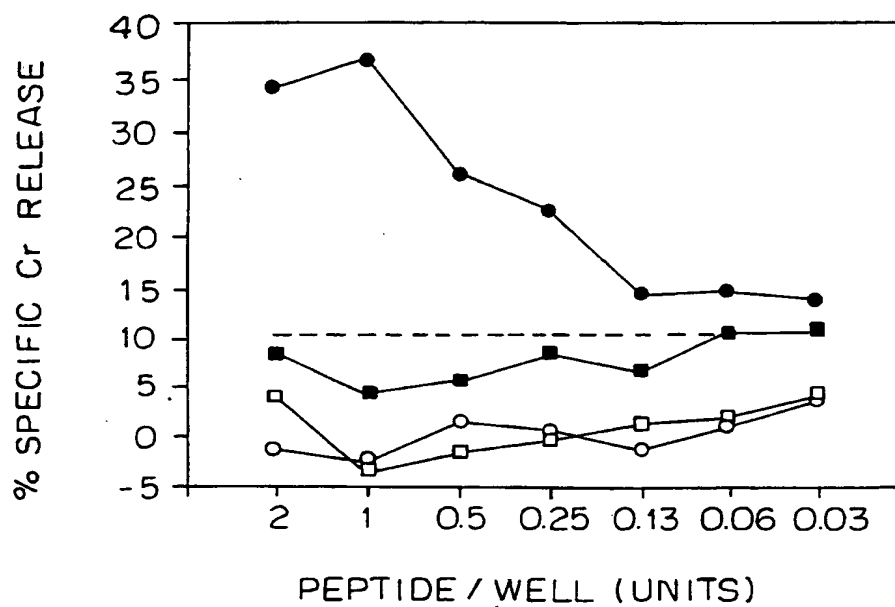


FIG. 2B



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FIG. 3



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FIG. 4A

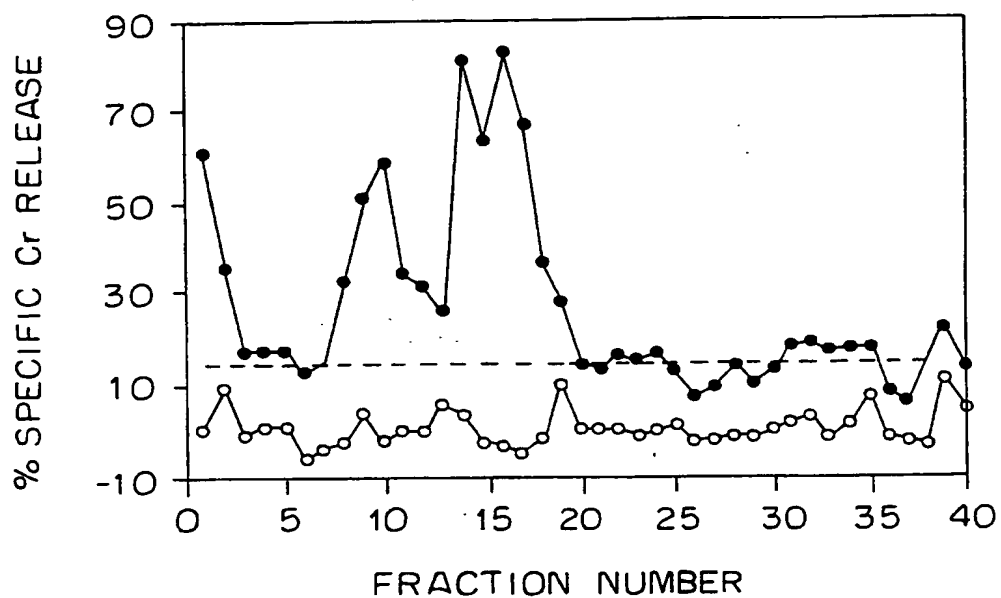
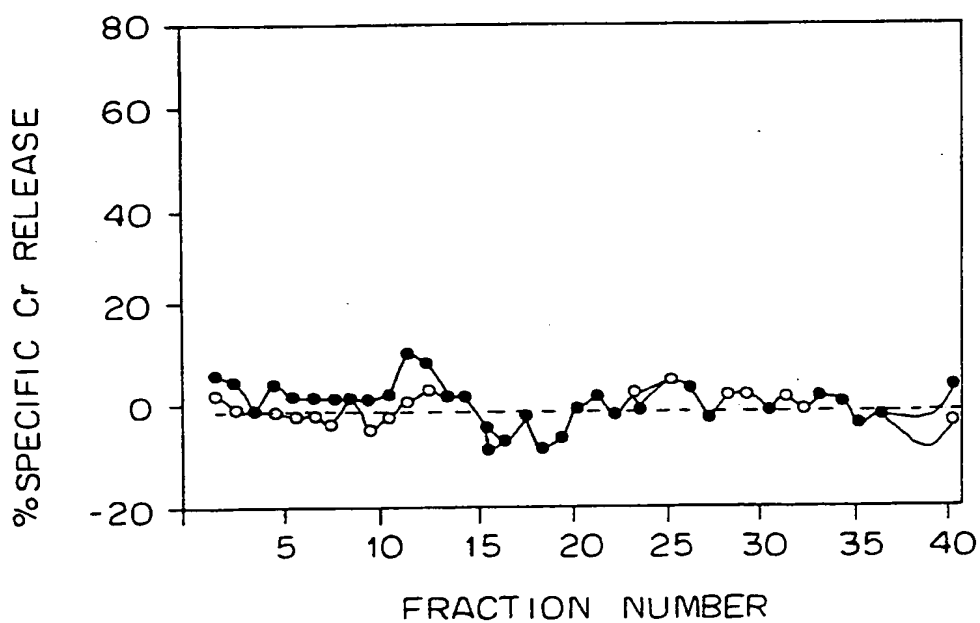


FIG. 4B



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FIG. 5A

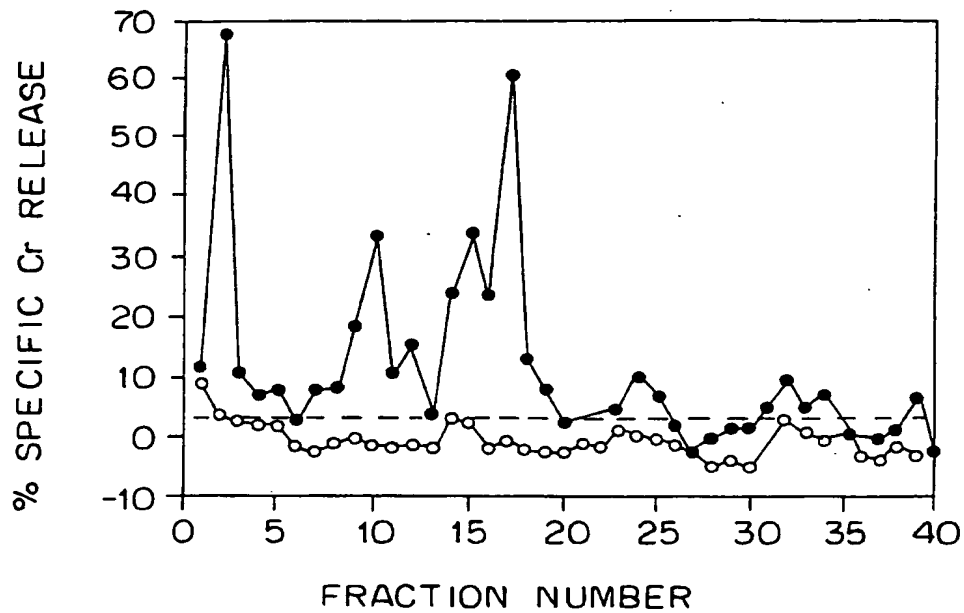
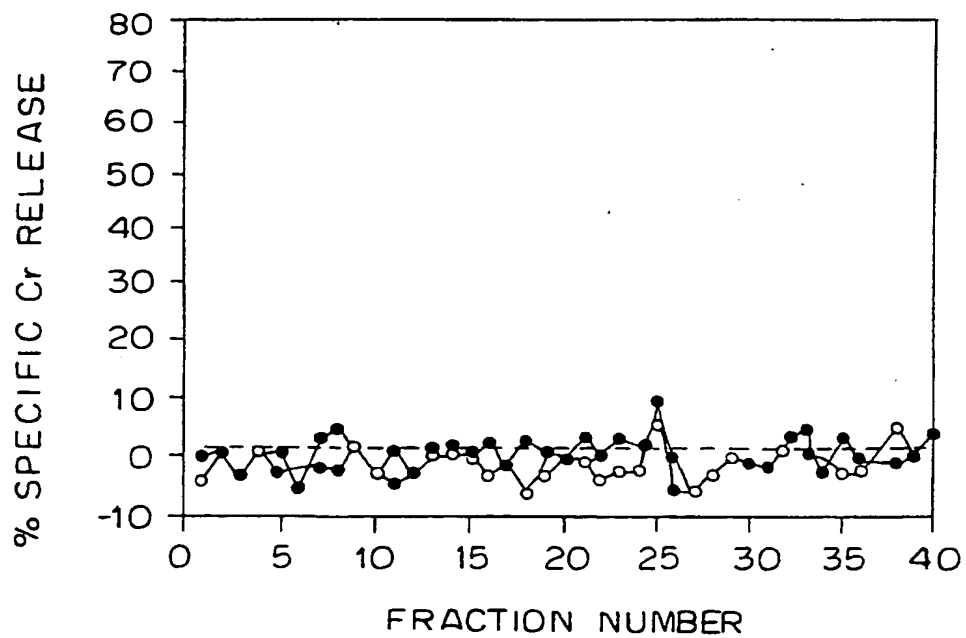


FIG. 5B



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FIG. 6

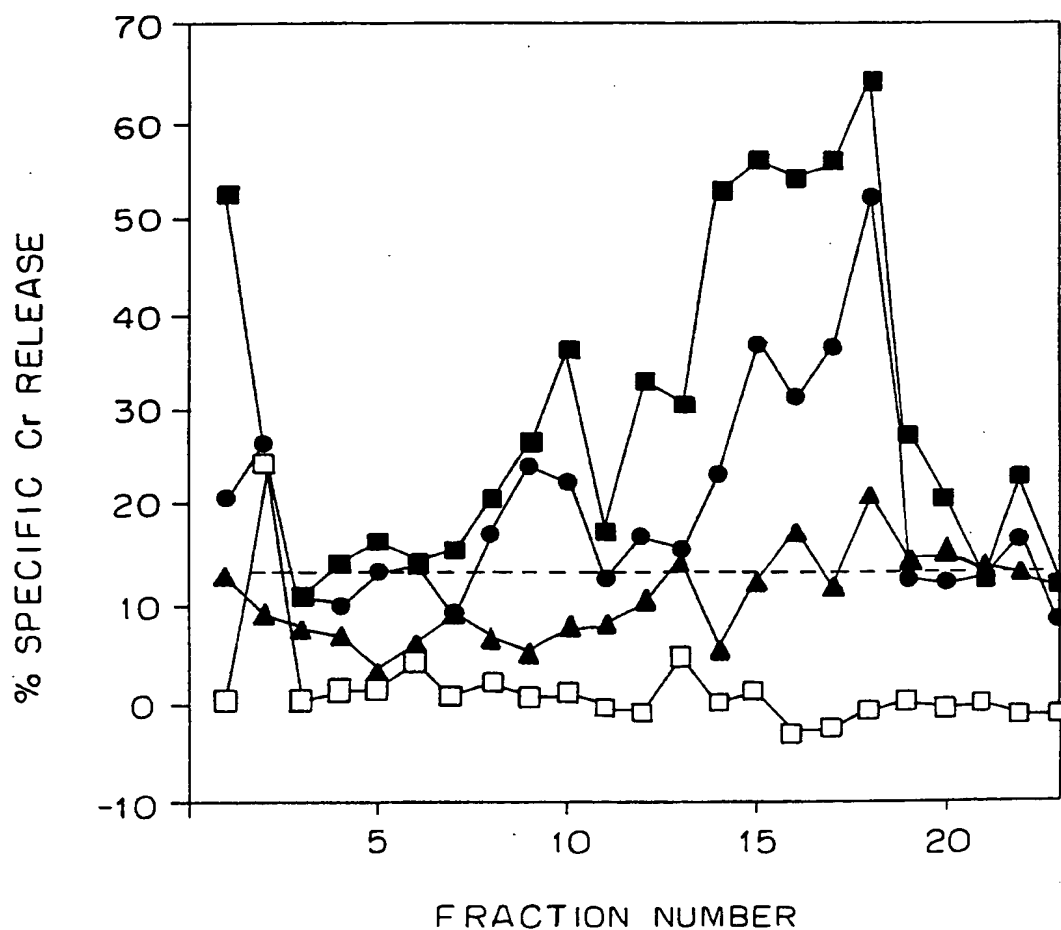


FIG. 7A

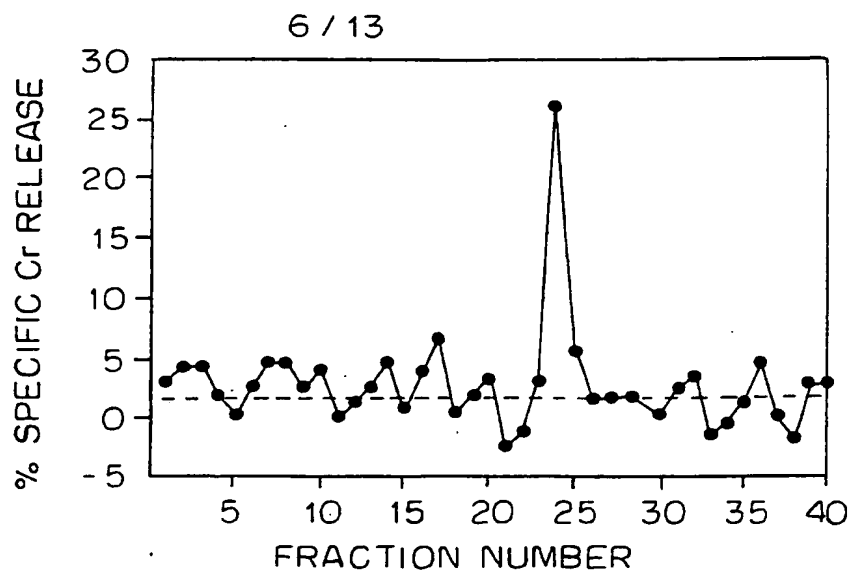


FIG. 7B

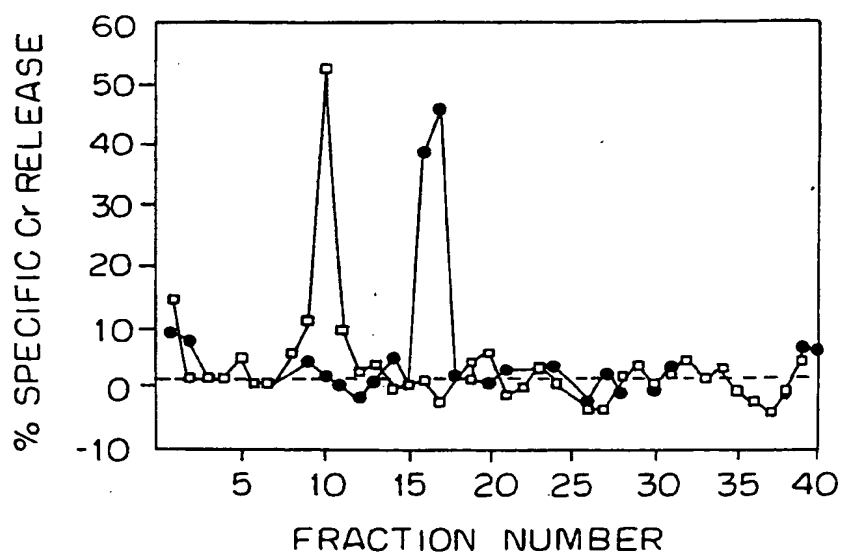
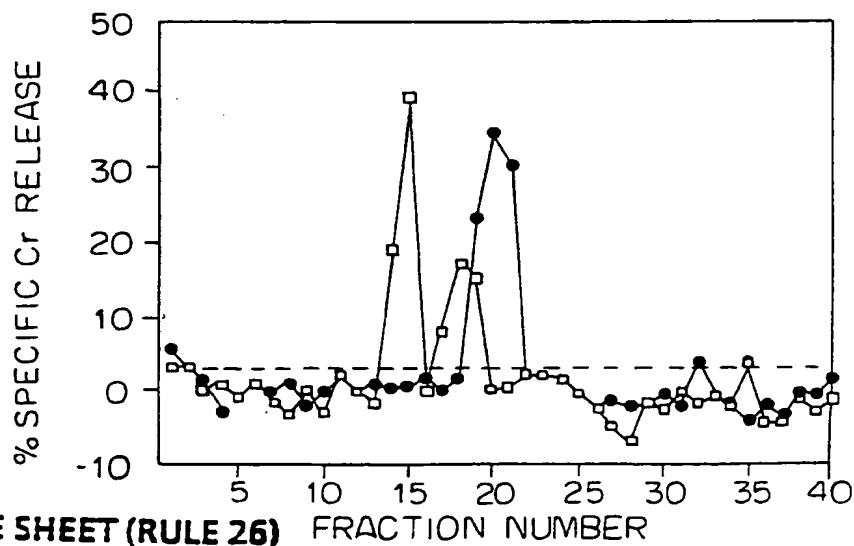


FIG. 7C



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FIG. 7D

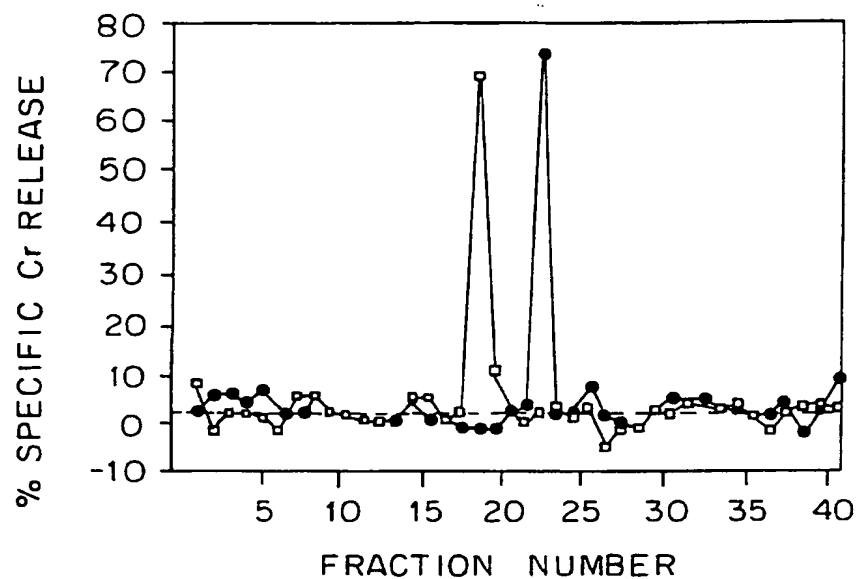
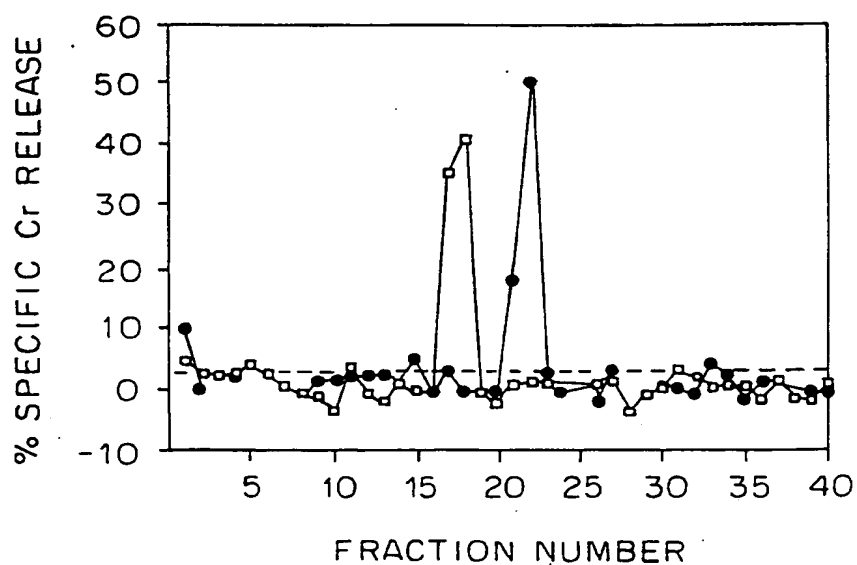


FIG. 7E



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FIG. 8A

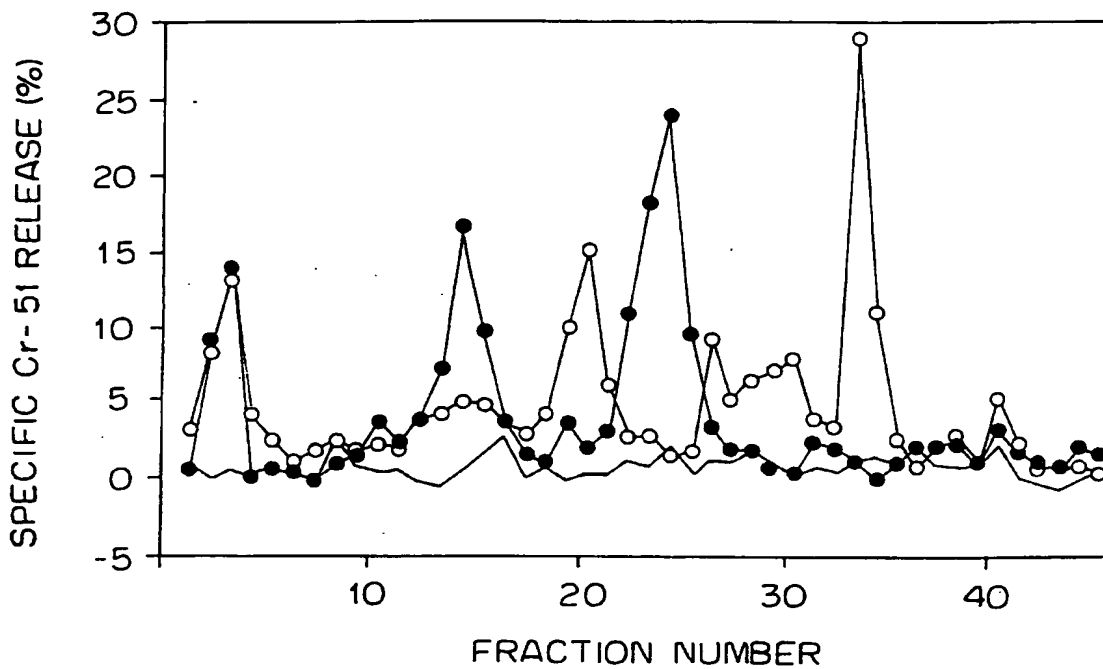
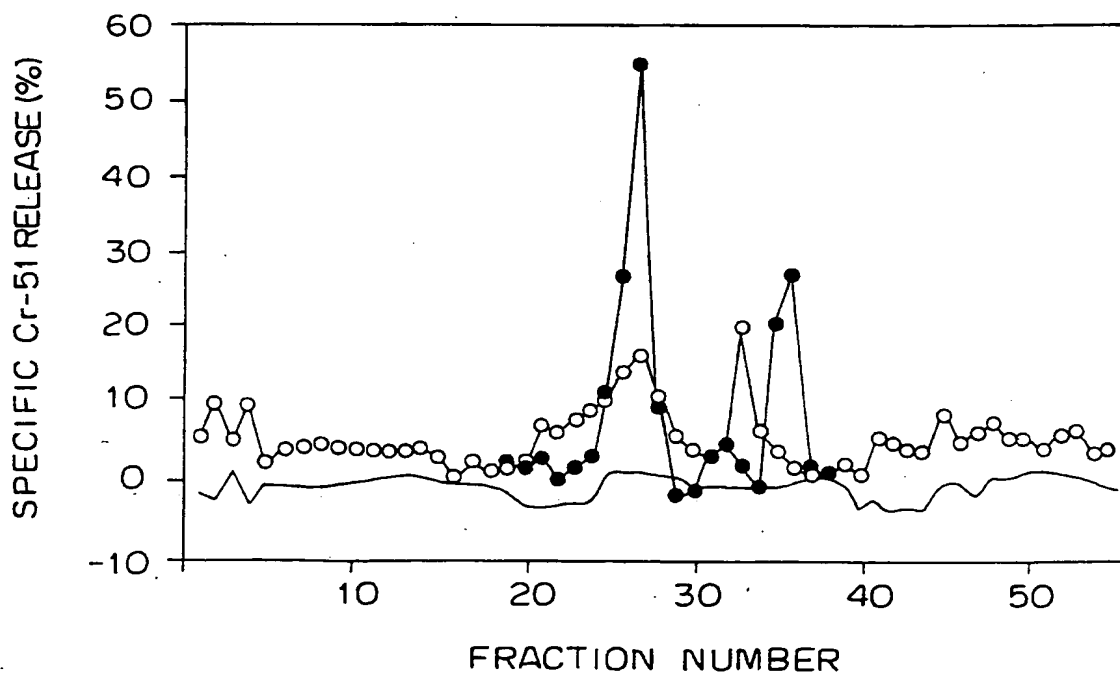


FIG. 8B



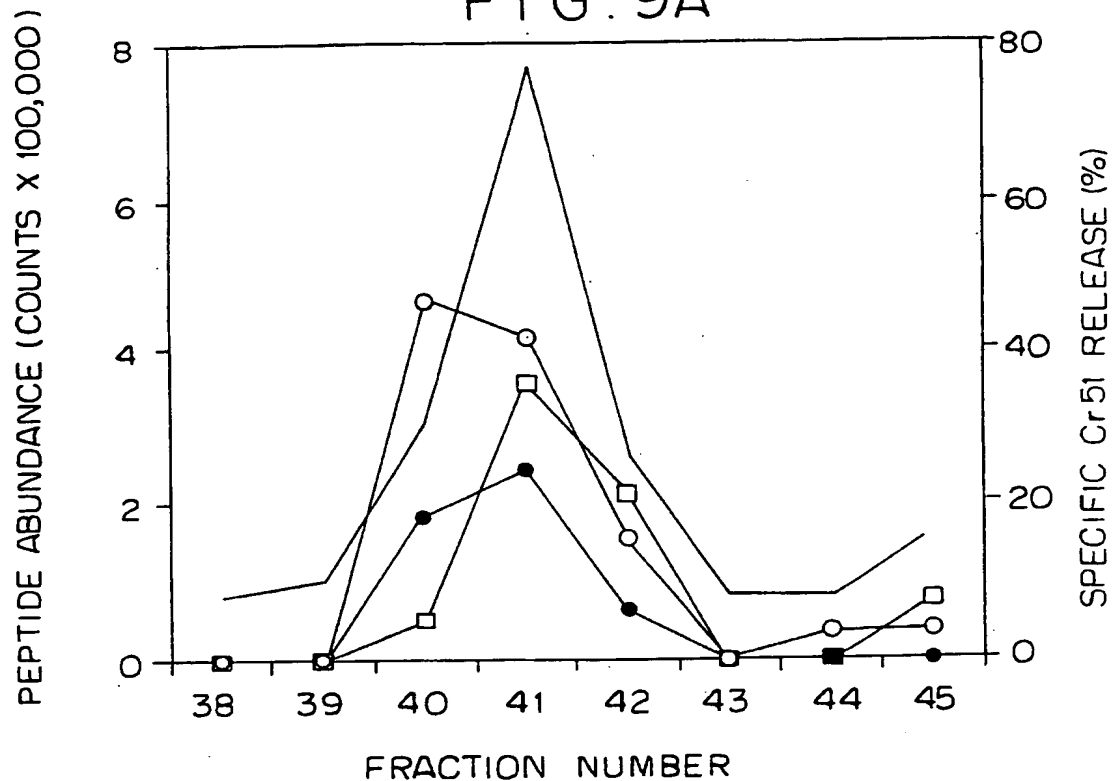
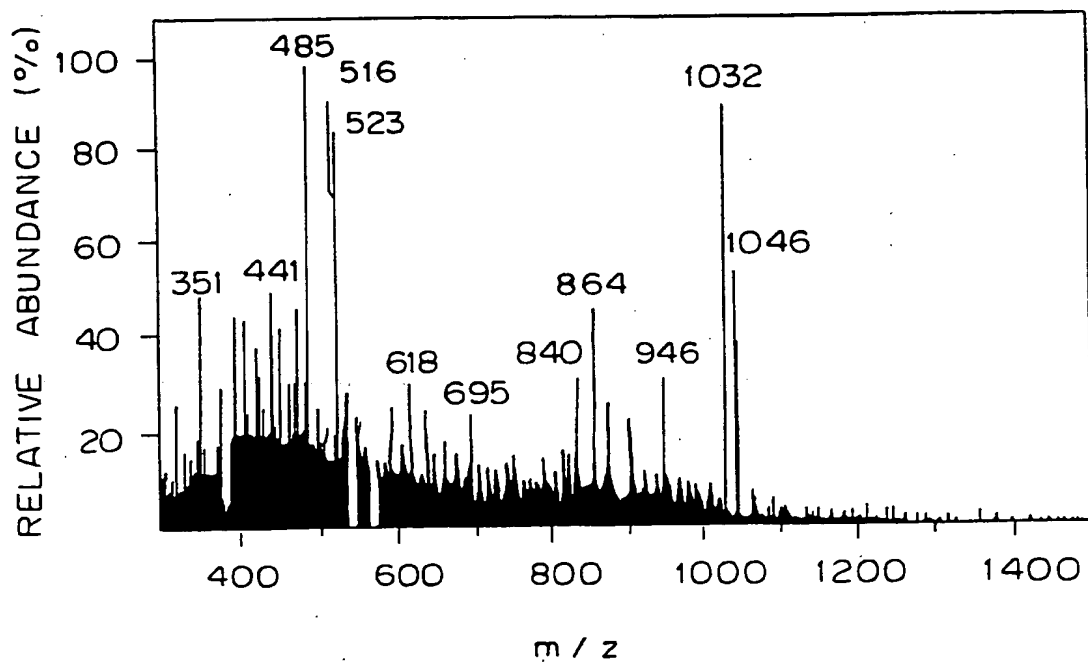
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FIG. 9A

FIG. 9B



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FIG. 10B

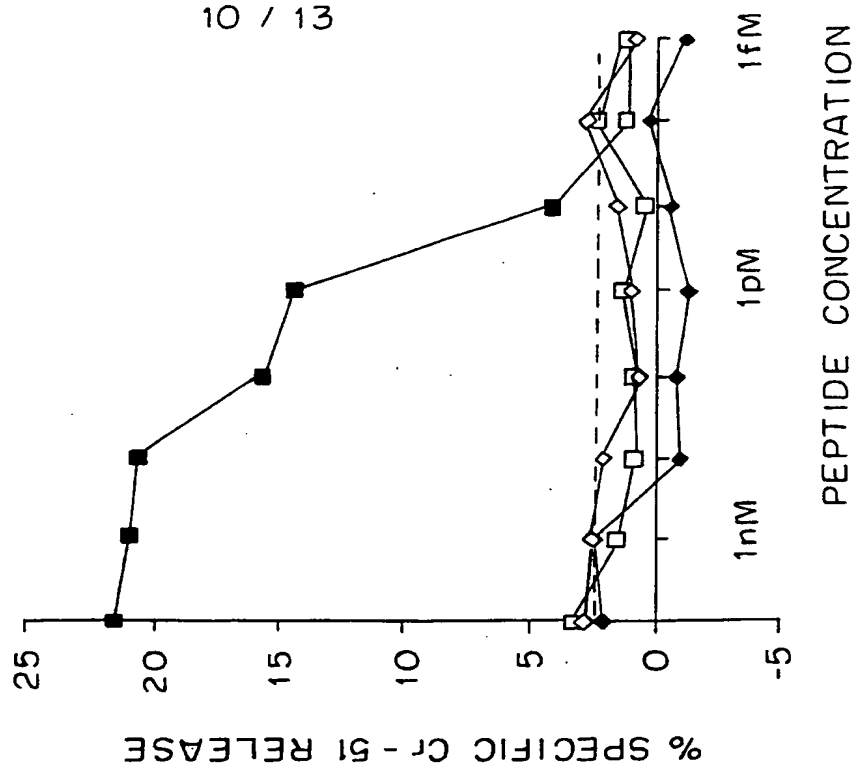
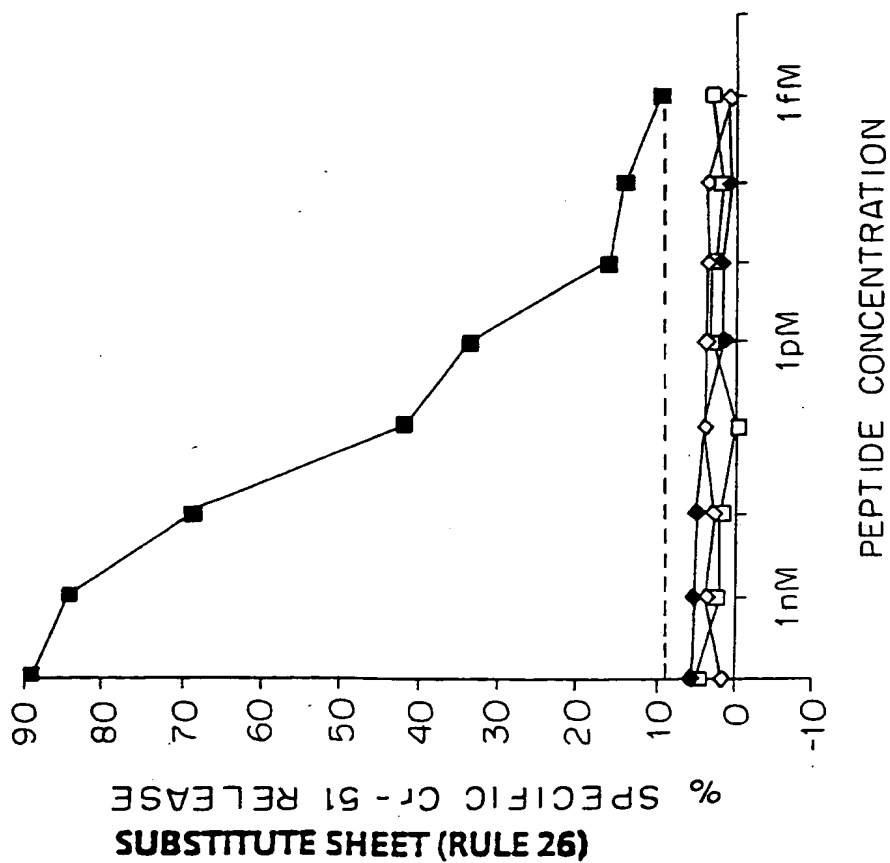
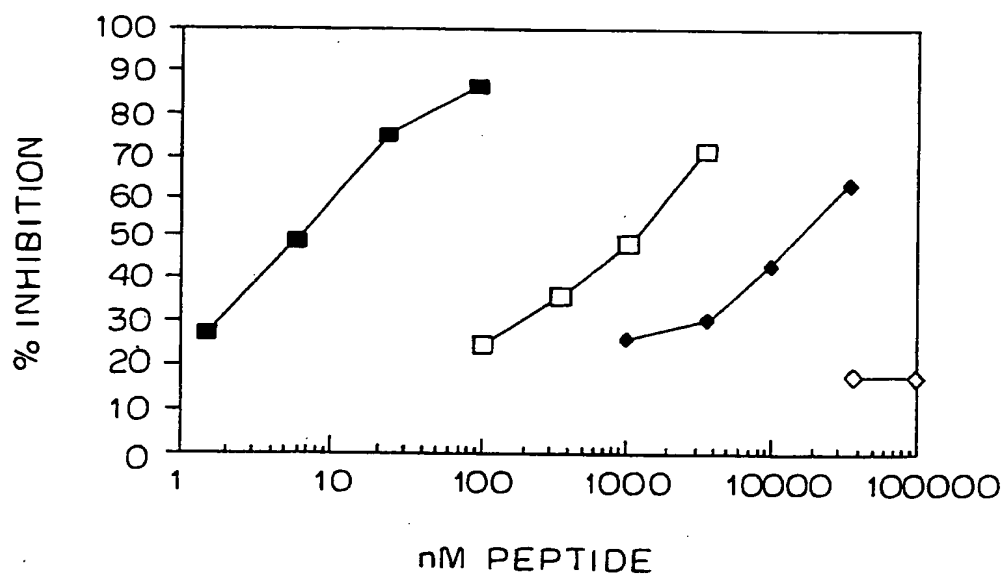


FIG. 10A



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FIG. 11



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FIG. 12

